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GLYCOLLATE PATHWAY INTERMEDIATES AND RELATED FOLATE METABOLISM IN GREENING BARLEY LEAVES

by

DAVID JOHN GIFFORD

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled GLYCOLLATE PATHWAY INTERMEDIATES AND RELATED FOLATE METABOLISM IN GREENING BARLEY LEAVES submitted by DAVID JOHN GIFFORD in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Biochemistry.

ABSTRACT

Interrelationships between glycollate and folate metabolism were examined at optimal light intensity (500 μeinsteins m^{-2} sec^{-1}) during de-etiolation of 6 day old *Hordeum vulgare* L. cv. Galt leaves. Emphasis was placed on the generation and metabolism of formate, and enzymes catalyzing these reactions were examined in cell-free extracts. The activity of glycollate : oxygen oxido-reductase E.C. 1.1.3.1 (glycollate oxidase), 5,10-methylenetetrahydrofolate : $NADP^+$ oxido-reductase E.C. 1.5.1.5 (5,10-methylenetetrahydrofolate dehydrogenase), formate : tetrahydrfolate ligase E.C. 6.3.4.3 (10-formyltetrahydrofolate synthetase), and the rate of enzymically coupled glyoxylate decarboxylation increased significantly during greening. Maximal activities were reached after 48 hrs of illumination. A potential carbon flow from glycollate → glyoxylate → formate → 10-formyltetrahydrofolate, which could account for ca. 20% of glycollate metabolized during greening was suggested by the enzyme data. Additional evidence for this pathway was provided by study of α-hydroxy-2-pyridinemethane sulphonate-treated leaves. Leaves greened for 24 hrs in the presence of the inhibitor contained less 10-formyltetrahydrofolate synthase activity, and ability to incorporate [^{3}H]- and [^{14}C]formate was reduced by up to 45%.

The metabolism of [^{3}H]formate was also examined during a 4 day greening period. In these epxeriments ^{3}H was more actively incorporated by sections of greening leaves than by etiolated controls. The rate of formate incorporation increased markedly during the first 48 hrs of greening, thus paralleling the enzyme data for formate generation and metabolism. Glycine, serine and aspartate were the only amino acids labelled

from [^{3}H]formate, with the former being the most important product during the initial 24 hrs of greening. The involvement of folate-dependent enzymes in this metabolism was demonstrated by the inhibition of [^{3}H]-formate incorporation in leaves greened for 24 hrs in the presence of aminopterin.

To examine glycine labelling in more detail, leaf sections were preincubated with iso-nicotinyl hydrazide prior to feeding. Glycine labelling was markedly decreased and its effect was related to inhibitor concentration. These treatments increased the pool size of glycine by 10-fold, indicating significant rates of turnover. The data suggested that glycine turnover from formate, via 5,10-methylenetetrahydrofolate : glycine hydroxymethyltransferase E.C. 2.1.2.10 (glycine synthetase).

To assess carbon flow from glycollate, labelled intermediates of the glycollate pathway were supplied to sections of leaves greened for 24 hrs. While [2-^{14}C]glycollate was rapidly incorporated into glycine, only low levels of ^{14}C were detected in serine. These tissues metabolized [1-^{14}C]- and [2-^{14}C]glycine to serine at rates indicative of low glycine cleavage and 5,10-methylenetetrahydrofolate : ammonia hydroxymethyltransferase E.C. 2.1.2.1 (serine hydroxymethyltransferase) activity. Additionally [3-^{14}C]serine was only slightly incorporated into the sugar fraction. Thus glycine appeared to be an important product of glycollate metabolism in partially greened leaves.

It is concluded that as etiolated leaves green there is a gradual activation and coordination of the partial reactions of the glycollate pathway. Throughout this period glycine assumes an important role as a metabolic product from both glycollate and formate.

ACKNOWLEDGEMENTS

I wish to thank Dr. E.A. Cossins for his advice, encouragement and insight throughout the course of this study. His editorial comments during the preparation of this thesis were both needed and appreciated.

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LIST OF ABBREVIATIONS*

C-1	:	one-carbon
THFA**	:	tetrahydrofolic acid
H_4 PteGlu**	:	tetrahydropteroylglutamate
10-HCO-H_4PteGlu***	:	N^{10}-formyltetrahydropteroylglutamate
5,10-CH_2-H_4 PteGlu	:	N^5,N^{10}-methylenetetrahydropteroylgutamate
5,10-CH=H_4 PteGlu	:	N^5,N^{10}-methenyltetrahydropteroylglutamate
RuDP carboxylase	:	Ribulose 1,5- bisphosphate carboxylase
P-glycollate	:	Phosphoglycollate
P-glycollate phosphatase	:	Phosphoglycollate phosphatase
PEP carboxylase	:	Phosphoenolpyruvate carboxylase
TPP	:	Thiamin pyrophosphate
3PGA	:	3 phosphoglyceric acid
ADP, ATP	:	Adenosine diphosphate, adenosine triphosphate
NAD (P)	:	nicotinamide-adenine dinucleotide (phosphate)
FMN	:	Flavin mono nucleotide
INH	:	isonicotinyl hydrazide
αHPMS	:	α-hydroxy-2-pyridinemethane sulfonate
dpm	:	disintegration per minute
μci	:	microcurie
EC ****	:	Enzyme Commission
Eight-day greened leaves	:	six day old etiolated leaves which have been greened for 2 days.
Eight-day etiolated leaves	:	six-day old etiolated leaves which have been held in the dark for 2 days.

S.E.M. : Standard error of the mean

* Other abbreviations commonly utilized in the text are given in the format acceptable for publication in the *Biochemical Journal* as listed in Vol. 153: 1-21 (1976).

** These two are synonymous.

*** The abbreviations used for tetrahydropteroylglutamic acid and its derivatives are those suggested by the IUPAC-IUB Commission in the *Biochemical Journal* Vol. 102: 15 (1967).

**** The recommendations (1972) of the commission on biochemical nomenclature were followed as published in *Enzyme nomenclature* (1973), Elsevier, Amsterdam.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

LIST OF TABLES CONTINUED

LIST OF FIGURES

LIST OF SCHEMES

INTRODUCTION

One-carbon derivatives of tetrahydrofolic acid (H_4PteGlu) act as co-enzymes in many metabolic reactions, including the formation of purines, pyrimidines, thymidylate, tetrapyrrole derivatives, and several amino acids (see excellent reviews by Blakley, 1969; Henderson, 1972; Flavin, 1975). The demands made on the one-carbon pool, to satisfy these folate-dependent syntheses depend upon the metabolic status of the cell. Thus actively growing cells have the greatest requirements for C-1 units. In addition these demands result in a need for increased pools of folate precursors. In general the metabolic activities of pathways associated with the generation of C-1 units can be expected to parallel those involved in their metabolism, with the demands of the cell for cellular constituents serving as the driving force. Consequently periods of development present unique opportunities for study of precursor-product relationships between associated biochemical pathways. One such period occurs when leaves green and acquire photosynthetic capability.

There have been extensive studies of the morphological and biochemical events which occur in etiolated leaves upon illumination. These events clearly involve the integrated activities of the nucleus, cytoplasm, mitochondria and plastids. The greening process also involves mobilization of reserves from other parts of the plant. (Beevers, 1976).

Etiolated seedlings undergo marked changes in growth-habit upon illumination (Beevers, 1976). In dicotyledons, hypocotyl or epicotyl growth is retarded and the plumular hook unfolds. In these species, leaf expansion after illumination is due to an increase in cell size, although some cell division may occur in young etiolated

leaves. Illumination of etiolated cereal seedlings causes the mesocotyl to stop elongating and the leaves unroll and become green. These light induced changes in morphology are mediated by phytochrome (Leopold and Kriedemann, 1975).

During greening leaf etioplasts undergo characteristic ultrastructural changes. In this process the prolamellar body is converted into "sheets", and thylakoids begin to develop leading finally to grana formation (Bradbeer *et al.*, 1977). In *Euglena* and *Zea mays* there is good evidence that proplastids and mitochondria are closely associated during chloroplast development (Schiff, 1970; Montes and Bradbeer, 1976). In addition Bradbeer *et al.*, (1977) noted that there appear to be synchronized changes in the inner membranes of plastids and mitochondria during greening. It was suggested that the mitochondria may be providing additional reducing power and high energy phosphate for the developing chloroplasts prior to the onset of photosynthesis.

Organelle studies of wheat and bean leaves (Feierabend and Beevers, 1972 b; Gruber *et al.*, 1973) suggest that peroxisomes also undergo morphological change during greening. It appears that light is required in the generation of peroxisomes from precursor particles. In addition Feierabend and Beevers (1972 b) have shown that peroxisomes develop normally when leaves are exposed to treatments which inhibit chlorophyll formation. They suggest therefore that although light is required for the production of both leaf peroxisomes and chloroplasts, the two processes are regulated independently. A similar conclusion has been drawn from studies of barley and other cereal leaves (Feierabend and Mikus, 1977). Developmental studies of wheat peroxisomes (Feiera-

bend 1975) indicate that the phytochrome system is involved. However these studies revealed an additional response to wavelengths which would not implicate this pigment system.

The expansion or unrolling of leaves in response to illumination is accompanied by a decrease in the pools of many soluble amino acids (Hendry and Stobart, 1977a). This is accompanied by rapid increases in synthesis of protein. (Chen *et al.*,1967; Murray *et al.*, 1973) chlorophyll (Huffaker, *et al.*, 1966; Graham *et al.*, 1970), RNA (Bradbeer, 1969; Poulson and Beevers, 1970) and folates (Spronk and Cossins, 1972). These increases imply *de novo* enzyme synthesis, or activation in response to light. In this regard considerable interest has focused on the effect of greening enzymes of the Calvin cycle and other photosynthetically related processes.

There is now good evidence for the *de novo* synthesis of RuDP carboxylase in response to greening in barley (Keller and Huffaker, 1967; Kleinkopf *et al.*, 1970), corn (Chen *et al.*,1967; Graham *et al.*, 1970) bean (Bradbeer, 1969), sorghum, wheat and oats (Graham *et al.*, 1970). Evidence for *de novo* synthesis of a variety of other chloroplastic enzymes, including PEP carboxylase in sorghum and corn (Graham *et al.*, 1970) has also been provided in some of these studies. The activity of the peroxisomal enzyme, glycollate oxidase, has long been known to be enhanced by light (Tolbert and Burris, 1950; Tolbert and Cohan, 1953), and there is good evidence that this may be phytochrome mediated (Klein, 1969). Developmental studies on bean (Murray *et al.*, 1973; Gruber *et al.*, 1973) and wheat (Feierabend and Beevers, 1972 a) have shown that other peroxisomal enzymes, associated with the glycollate and glycerate pathways of C_3 plants, undergo rapid *de novo* synthesis

in response to greening. Until the present study the effect of greening on enzymes associated with folate metabolism had not been assessed.

Implicit in these data is a high degree of integration between organelle development and the coordination of enzymes into biosynthetic pathways during greening. Particular interest has focused on the development of photosynthetic pathways. In this regard Tamàs *et al.*, (1970) suggested that illumination of etiolated barley leaves resulted in successive activation of three distinct photosynthetic processes; firstly, a light-stimulated ß-carboxylation, secondly the Calvin cycle and thirdly, the glycollate pathway.

Photorespiration and the glycollate-glycerate pathways of C_3 plants.

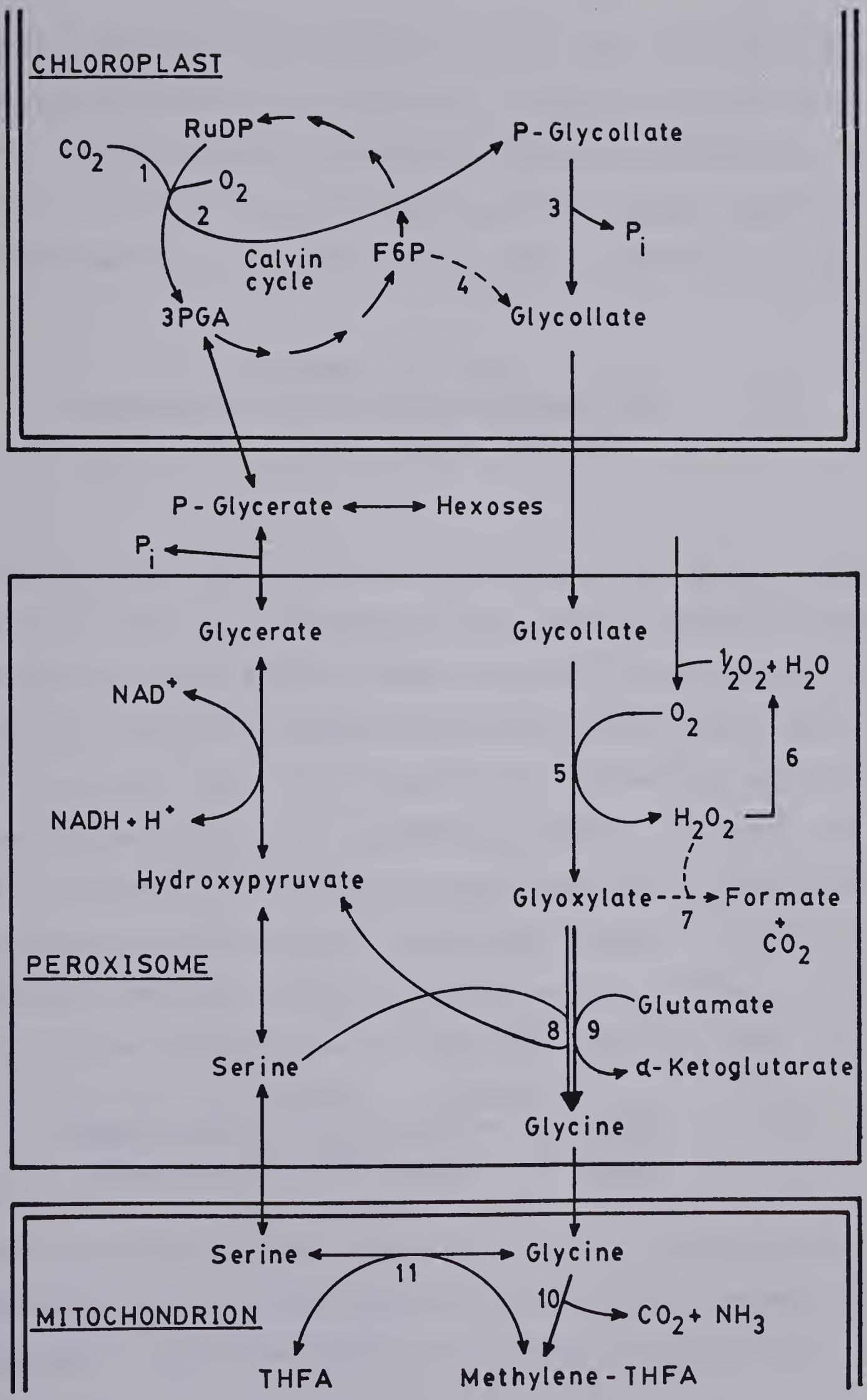
Illuminated green leaves of C_3 plants release a proportion of recently fixed CO_2 in a light-dependent process known as photorespiration. This process occurs by biochemical reactions different from "dark" mitochondrial respiration, and can result in as much as 50% of recently fixed CO_2 being lost as photorespiratory CO_2 (Zelitch, 1975). Net assimilation in species exhibiting high rates of photorespiration will therefore be significantly reduced. In addition to C_3 plants, both algae and C_4 species exhibit characteristics of photorespiration (for reviews on the nature of photorespiration in algae and C_4 plants see Challet and Ogren, 1975; Chollet, 1976; Schnarrenberger and Fock, 1976).

Photorespiration is closely related to the formation of glycollic acid, and its subsequent oxidative decarboxylation (Jackson and Volk, 1970; Tolbert 1971; Tolbert and Ryan, 1976). Of the many hypotheses that have been proposed for glycollate formation (see

Scheme 1. The glycollate-glycerate pathways in C_3 plants.

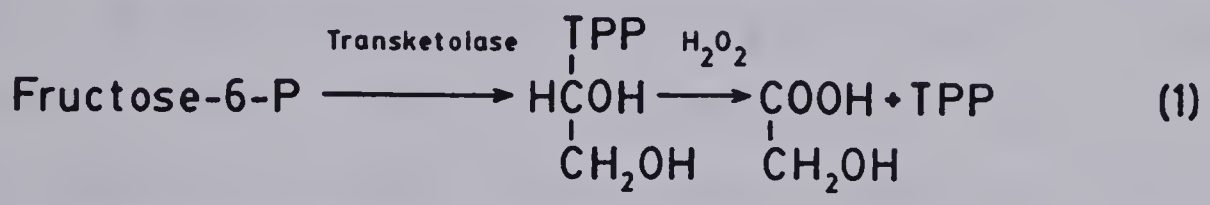
Reaction number	Trivial Enzyme Name	Systematic Enzyme Name	E.C. Number
1	Ribulosediphosphate carboxylase	3-Phospho-D-glycerate carboxy-lyase	4.1.1.39
2	Ribulosediphosphate oxygenase		
3	Phosphoglycollate phosphatase	2-Phosphoglycollate phosphohydrolase	3.1.3.18
4	DHETPP oxidation (non-enzymatic)		
5	Glycollate oxidase	Glycollate:oxygen oxido-reductase	1.1.3.1
6	Catalase	hydrogen peroxide:hydrogen peroxide oxido-reductase	1.11.1.6
7	Glyoxylate decarboxylation (non-enzymatic)		
8	Serine-glyoxylate aminotransferase	L-Serine:glyoxylate aminotransferase	2.6.1.45
9	Glycine aminotransferase	Glycine:2-oxoglutarate aminotransferase	2.6.1.4
10	Glycine synthase	5,10-Methylenetetrahydrofolate:glycine hydroxymethyltransferase	2.1.2.10
11	Serine hydroxymethyltransferase	5,10-Methylenetetrahydrofolate:ammonia hydroxymethyltransferase	2.1.2.1

(Adapted from Chollet and Ogren, 1975; Tolbert and Ryan, 1976; Halliwell, 1978).


CHLOROPLAST
RuDP
CO_2
1
O_2
2
Calvin cycle
3PGA
F6P
4
P-Glycollate
3
P_i
Glycollate
P-Glycerate ⟷ Hexoses
P_i
Glycerate
Glycollate
$\frac{1}{2}O_2 + H_2O$
NAD^+
$NADH + H^+$
O_2
5
6
H_2O_2
Hydroxypyruvate
Glyoxylate
7
Formate
CO_2
PEROXISOME
Glutamate
8
9
Serine
α-Ketoglutarate
Glycine
Serine ⟷ Glycine
11
10
$CO_2 + NH_3$
MITOCHONDRION
THFA
Methylene-THFA

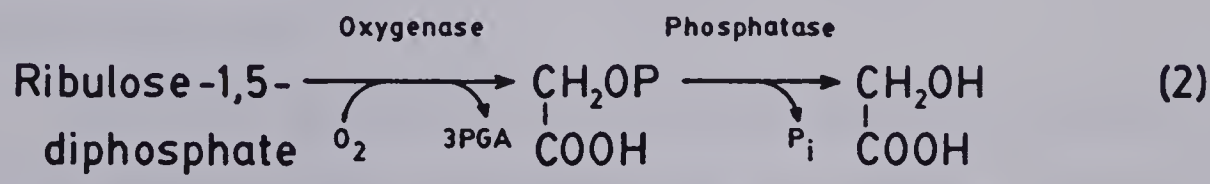
Tolbert, 1973 and Zelitch, 1975 for reviews), biosynthesis via RuDP carboxylase/oxygenase and transketolase have received most attention.

Glycollate can be formed in a scheme involving the non-enzymic oxidation of a transketolase C_2-addition complex, equation 1 (Whittingham *et al*, 1967; Gibbs, 1969; scheme 1, reaction 4). There



(1)

is some evidence that glycollate can be formed *in vivo* by this mechanism (Shain and Gibbs, 1971; Robinson and Gibbs, 1974). However this proposal has received criticism since it fails to account for the observed incorporation of $^{18}O_2$ into the carboxyl group of glycollate (Andrews *et al.*, 1971), nor does it account for P-glycollate formation and the presence of a very active chloroplastic P-glycollate phosphatase (Richardson and Tolbert, 1961). A more satisfactory mechanism, and one which is rapidly gaining acceptance as the major route of glycollate synthesis in C_3 plants, is glycollate formation via RuDP carboxylase, equation 2 (scheme 1, reaction 2; for reviews see Andrews and Lorimer, 1978; Halliwell, 1978). In this



(2)

scheme glycollate is formed from phosphoglycollate, produced by the oxygenase activity of RuDP carboxylase (Tolbert, 1973). In addition to the above this mechanism satisfies other criteria associated with

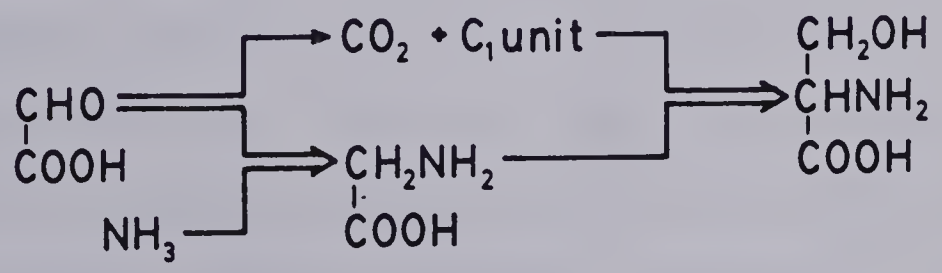
glycollate formation and photorespiration, including a light requirement and competition between O_2 and CO_2 resulting in more glycollate formation at high O_2 concentrations (Tolbert and Ryan, 1976).

Current studies (see Tolbert and Ryan, 1976 and Scharrenberger and Fock, 1976) suggest that glycollate is metabolized in a reaction sequence involving chloroplasts, peroxisomes and mitochondria, scheme 1. In this scheme carbon flow to glycine occurs via the glycollate pathway. Serine, formed from glycine in the mitochondria, can be further metabolized to 3PGA via the reversible reactions of the glycerate pathway. The 3PGA can either re-enter the carbon reduction cycle in the chloroplast or be converted to hexoses via cytoplasmic reactions that are gluconeogenic. It should be noted that the glycerate pathway may also provide a mechanism for serine and glycine synthesis from glycerate in tissues where the glycollate pathway is absent or inoperative (Cheung *et al.*, 1968).

There is some evidence (Bird *et al.*, 1972 a, b) that the conversion of glycine to serine in tobacco leaf mitochondria is coupled to ATP synthesis. A similar finding has been reported for *Euglena* (Collins *et al.*, 1975). At present the significance of these findings, in relation to *in vivo* ATP production outside the chloroplast, has yet to be fully ascertained.

While the production of CO_2 during photorespiration is associated with the reactions of the glycollate pathway, there has been much speculation in the literature concerning the immediate substrate of photorespiratory CO_2 (see Halliwell, 1978 for a review). In this regard, both glycine and glyoxylate appear to make some contribution. Plant mitochondria convert glycine into serine and CO_2 via the combined action of glycine synthase and

serine hydroxymethyltransferase (Tolbert, 1971; Prather and Sisler, 1972; Clandinin and Cossins, 1975; scheme 1, reactions 10 and 11). As the CO_2 released in this conversion arises indirectly from the carboxyl group of glycollate,glycine decarboxylation is considered by many workers (Kisaki and Tolbert, 1970; Kisaki *et al.*, 1971 a; Kisaki *et al.*, 1971 b) to be the major source of photorespiratory CO_2. There is however good evidence that the decarboxylation of glyoxylate also makes a significant contribution to the release of CO_2 during photorespiration (Zelitch, 1972 a; Waidyanatha *et al.*, 1975; scheme 1, reaction 7). Studies *in vitro* have demonstrated that chloroplastic (Zelitch, 1972 b) and peroxisomal (Halliwell and Butt, 1974) preparations can carry out this reaction. Consistent with these finding are the results of $^{14}CO_2$ studies on sunflower leaf discs (Mahon *et al.*, 1974; Canvin *et al.*, 1976). These latter workers have suggested that in sunflower leaves the production of CO_2 and glycine is from a common precursor, glyoxylate, equation 3. In this scheme there is no glycine cleavage, the CO_2 being produced solely from the carboxyl group



(3)

of glyoxylate. This conclusion must be viewed with caution however, in the light of the considerable evidence from *in vitro* studies reviewed above, which

support the conversion of glycine to CO_2 and serine.

The glycollate pathway, and the generation and metabolism of C-1 units in plants.

Unlike animals and bacteria, plants cannot derive C-1 units *de novo* through purine and histidine catabolism (Henderson, 1972). Consequently it is generally believed that in plants, the major biological sources of C-1 units are glycine, serine and formate. The partial reactions of the glycollate pathway clearly include two of of these potential folate precursors, glycine and serine and a third, formate has been strongly implicated (scheme 1, reaction 7). As noted earlier, the predominant flow of carbon through the glycollate pathway is to carbohydrate via serine. However, during greening it is logical to assume that some of the carbon flow would be diverted to generate C-1 units needed to support a variety of folate-dependent syntheses. Scheme 2 summarizes the reactions involved in C-1 unit synthesis from these precursors.

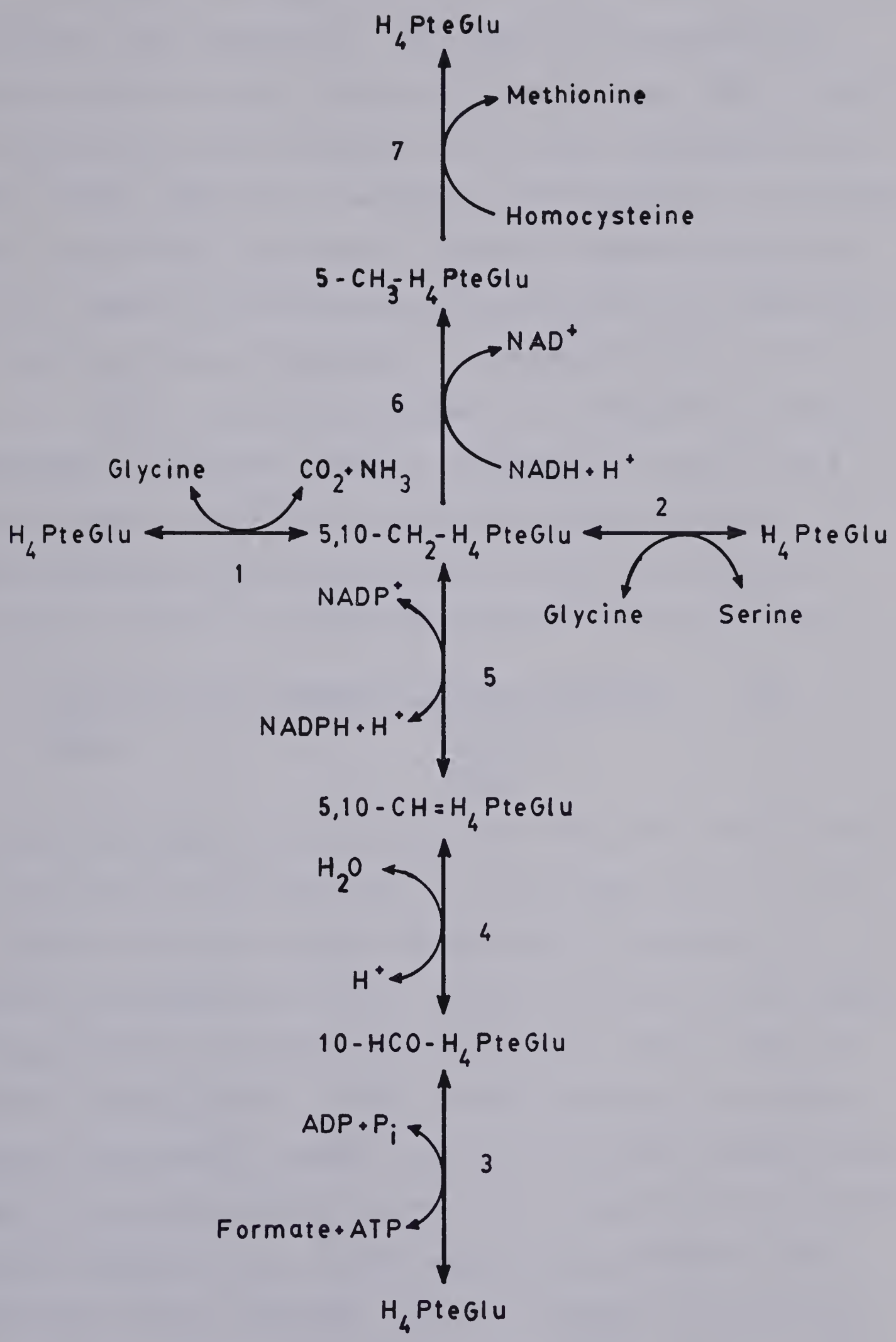
In the glycollate pathway serine is believed to be generated in a sequence involving two folate dependent reactions (scheme 1, reactions 10 and 11). The initial step in this scheme is the cleavage of glycine by glycine synthase to give 5,10-methylenetetrahydrofolate, CO_2 and ammonia, equation 4 (scheme 2, reaction 1). The reaction

$$\overset{\times}{C}H_2NH_2(COOH) + H_4PteGlu + NAD^+ \longleftrightarrow 5,10\text{-}\overset{\times}{C}H_2\text{-}H_4PteGlu + CO_2 + NH_3 + NADH + H^+ \qquad (4)$$

requires pyridoxal-5'-phosphate and there is good evidence for it in plants (McConnell, 1964; Cossins and Sinha, 1966; Kisaki *et al*.,

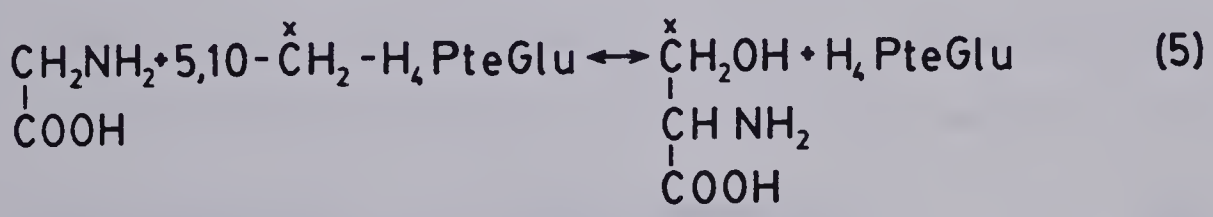
Scheme 2. The major reactions for the production of one-carbon units, and the interconversion of folate derivatives.

Reaction No.	Trivial enzyme Names	Systematic Enzyme Name	E.C. Number
1	Glycine synthase	5,10-Methylenetetrahydrofolate:ammonia hydroxymethyltransferase	2.1.2.10
2	Serine hydroxymethyltransferase	5,10-Methylenetetrahydrofolate:glycine hydroxymethyltransferase	2.1.2.1
3	10-Formyltetrahydrofolate synthetase or 10-HCO-H_4PteGlu synthetase	Formate:tetrahydrofolate ligase	6.3.4.3
4	5,10-Methenyltetrahydrofolate cyclohydrolase or 5,10-CH=H_4PteGlu cyclohydrolase	5,10-Methenyltetrahydrofolate 5-hydrolase	3.5.4.9
5	5,10-Methylenetetrahydrofolate dehydrogenase or 5,10-CH_2-H_4 PteGlu dehydrogenase	5,10-Methylenetetrahydrofolate:$NADP^+$ oxido-reductase	1.5.1.5
6	5,10-Methylenetetrahydrofolate reductase	5-Methyltetrahydrofolate:NAD^+ oxido-reductase	1.1.1.68
7	Tetrahydrofolate methyltransferase	5-Methyltetrahydrofolate:L-homocysteine methyltransferase	2.1.1.13


H4PteGlu
Methionine
7
Homocysteine
5-CH3-H4PteGlu
NAD+
6
Glycine
CO2+NH3
NADH+H+
2
H4PteGlu
5,10-CH2-H4PteGlu
H4PteGlu
1
NADP+
Glycine
Serine
5
NADPH+H+
5,10-CH=H4PteGlu
H2O
4
H+
10-HCO-H4PteGlu
ADP+Pi
3
Formate+ATP
H4PteGlu

1971 a,b; Clandinin and Cossins, 1972; Prather and Sisler, 1972; Woo and Osmond, 1976; Moore *et al*., 1977) where it is believed to be tightly coupled to serine synthesis (Tolbert and Ryan, 1976). However in animals and bacteria (Kawasaki, *et al*., 1966; Yoshida and Kikuchi, 1971; Kikuchi, 1973) this mitochondrial decarboxylation is an important route for C-1 unit biosynthesis. A similar situation may occur in plants. However the contribution of this reaction to the generation of methylene folates in autotrophs has not been assessed in detail.

Within the glycollate pathway it is envisaged that serine hydroxymethyltransferase catalyses the formation of serine, from a second molecule of glycine, in a reversible reaction requiring 5,10-methylenetetrahydrofolate and pyridoxal-5'-phosphate, equation 5 (scheme 2, reaction 2). Serine hydroxymethyltransferase has been



detected in a wide variety of plant tissues (Hauschild, 1959; Wilkinson and Davies, 1960; Mazelis and Liu, 1967; Kisaki *et al*., 1971 a). In addition the enzyme has been demonstrated in chloroplasts of spinach and tobacco leaves (Kisaki *et al*., 1971 a) and in mitochondria of pea cotyledons (Clandinin and Cossins, 1972), spinach leaves and leaves of *Vicia faba* (Woo, 1979). Although the roles of the chloroplastic and cytosolic enzymes are not clear, there is good evidence that it is the mitochondrial enzyme which is involved in the glycollate pathway (Tolbert, 1971). In this context no net methylene folate generation would be envisaged. This is in contrast to bacterial and animal systems (Nakano *et al*.,1969; Fujioka, 1969). In animals the

mitochondrial reaction constitutes a major route for C-1 unit generation (Wright, 1955; Blakley, 1969). Examination of the literature shows however that little is known about the role of this enzyme in the general folate metabolism of plants.

In addition to the generation of C-1 units at the methylene level of oxidation *in vivo* studies (Tolbert, 1955; McConnell and Bilinski, 1959; Cossins and Sinha, 1965; Bowman and Rohringer, 1970; Kent, 1972) have suggested that plants can generate formyl folates via the activation of formate as a C-1 unit. In this regard glycollate pathway intermediates may play a role since there is evidence for their involvement in formate production. Tobacco leaf extracts (Tolbert *et al.*, 1949) and spinach-beet peroxisomes (Halliwell and Butt, 1974) catalyse the formation of formate from the C-2 of glycollate in a reaction involving oxygen uptake and the formation of glyoxylate as an intermediate, equations 6 and 7.

$$\overset{\times}{C}H_2OH\cdot COOH + O_2 \longrightarrow \overset{\times}{C}HO\cdot COOH + H_2O_2 \qquad (6)$$

$$\overset{\times}{C}HO\cdot COOH + H_2O_2 \longrightarrow H\overset{\times}{C}OOH + CO_2 \qquad (7)$$

Halliwell and Butt (1974) have proposed a similar mechanism for formate production from the C-2 of glyoxylate by spinach and spinach-beet peroxisomes. In this system glyoxylate is oxidised non-enzymically by hydrogen peroxide to formate and CO_2 (scheme 1, reaction 7).

In addition to leaf peroxisomes, plant mitochondria and chloroplasts are also capable of generating formate from the C-2 of glyoxylate. In tobacco leaf mitochondria, formate is formed in a reaction requiring oxygen, thiamine pyrophosphate, and a divalent metal ion (Prather and Sisler, 1972). A similar reaction has been reported for illuminated envelope-free spinach chloroplasts (Zelitch, 1972 b).

Davies and Corbett (1969) have shown that pyruvic decarboxylases of a variety of plant tissues will convert glyoxylate to formaldehyde, which can then be oxidised to formate by hydrogen peroxide. However the significance of this to *in vivo* formate production in relation to the glycollate pathway has not been assessed.

Carbon dioxide may also be a potential precursor of formate in higher plants. For example, formate was the first stable product of CO_2 fixation in chloroplasts isolated from green potato peelings (Ramaswany *et al.*, 1976). Furthermore Kent (1972) has claimed a direct reduction of CO_2 to formate in the leaves of *Vicia faba*. The enzymic pathway of formate production in these cases has not been clearly established, but Kent (1972) proposed a reaction (equation 8) involving a formate-CO_2 couple with an oxidation-reduction potential of -420 mv. He suggested that ferredoxin, with a redox potential of -430mv could be involved,

$$H---X_{(red)} + CO_2 \longrightarrow HCOO^- + X_{(ox)} \qquad (8)$$

noting that such a system has been detected in *Clostridium pasteurianum* (Tagawa and Arnon, 1962). In other bacteria formate may arise by reverse action of NAD^+- or $NADP^+$-linked formic dehydrogenases (Thauer, 1972, 1973 ; Ruschig *et al.*, 1976). However, in leaf tissues, $NADP^+$-coupled formic dehydrogenases have not been detected and activity of the corresponding NAD^+-coupled enzyme tends to be very low (Halliwell, 1974).

Activation of formate as a C-1 units involves formyltetrahydrofolate synthetase (scheme 2, reaction 3), which catalyses the production of 10-formyltetrahydrofolate in a reversible reaction requiring ATP, (equation 9). The enzyme has been detected in a wide variety of plant

species (Hiatt, 1965a; Iwai *et al.*, 1967; Halliwell, 1973; Crosti, 1974),

$$H\overset{\times}{C}OOH + H_4PteGlu + ATP \longleftrightarrow 10\text{-}H\overset{\times}{C}O\text{-}H_4PteGlu + ADP + P_i \quad (9)$$

and is well documented for many other organisms (Blakley, 1969) where it represents the major route of formyl folate synthesis. However the activity of the enzyme is low or not detected in many bacteria including *Escherichia coli* where formyl folate is derived from methylene folate (Harvey and Dev, 1975). In contrast bacteria which utilize purines for growth have higher synthetase levels (Decker *et al.*, 1970; Barker, 1972). Here it represents a mechanism for ATP generation.

Fractionation of plant extracts has shown the synthetase to be mainly cytoplasmic (Hiatt,1965a; Iwai *et al.*,1967; Halliwell, 1973; Crosti, 1974). However the enzyme has been found associated with spinach chloroplasts (Crosti, 1974) and the mitochondria of spinach (Crosti, 1974) and peas (Clandinin and Cossins, 1972). To date, evidence for the enzyme in leaf peroxisomes is lacking.

Once formed most folate derivatives are freely interconvertible through enzyme mediated reactions, scheme 2; the driving force in this scheme being the demand for C-1 units at a particular oxidation level. In this regard the interconversion of C-1 units between the formyl and methylene levels of oxidation requires the concerted action of two enzymes, namely cyclohydrolase and methylenetetrahydrofolate dehydrogenase (scheme 2, reactions 4 and 5 respectively). These enzymes have been studied in mammalian tissues and a variety of microorganisms (Blakley, 1969). They are present also in many higher plant tissues (Wong and Cossins, 1966; Suzuki and Iwai, 1973; Cossins *et al.*,1972). In beef liver the hydration of methenyltetrahydrofolate to formyltetrahydrofolate via the cyclohydrolase is usually favoured (Greenberg, 1971).

Suzuki and Iwai (1973) have reported similar findings for peas. However the ubiquitous presence of formyltetrahydrofolate synthetase in plants, coupled with the incorporation of formate into typical products of folate metabolism (Cossins and Sinha, 1965; Kent, 1972) argues for the reverse reaction in autotrophs. Partial purification of methylene-tetrahydrofolate dehydrogenase from pea seedlings has been described (Cossins *et al.*,1970), and some properties of this enzyme have been investigated.

Studies of yeast (Paukert *et al.*,1977) and mammalian liver (Paukert *et al.*, 1976; Tan *et al.*,1977) show that both of the above enzyme activities are closely associated with formyltetrahydrofolate synthetase. However at present there have been no reports of an analogous multi enzyme complex in higher plants.

Methylenetetrahydrofolate can be further reduced to the methyl level of oxidation by methylenetetrahydrofolate reductase (scheme 2, reaction 6). This is a key reaction in the biogenesis of methyl groups *de novo*, since the methyl derivative is required in transmethylations resulting in methionine (Dodd and Cossins, 1969, 1970; scheme 2, reaction 7) and S-adenosylmethionine (Mudd, 1960; Cantoni, 1965) biosynthesis. Once formed S-adenosylmethionine can act as a methyl donor in a variety of reactions in plant tissues. These include the methylation of t-RNA (Stone and Cherry, 1972; King and Chapman, 1973), lignin, pectin, chlorophyll, quinones and ferulic acid (Byerrum *et al.*,1954; Sato *et al.*, 1958; Radmer and Bogorad, 1967; Threlfall *et al.*,1967; Poulton and Butt, 1975).

The present study.

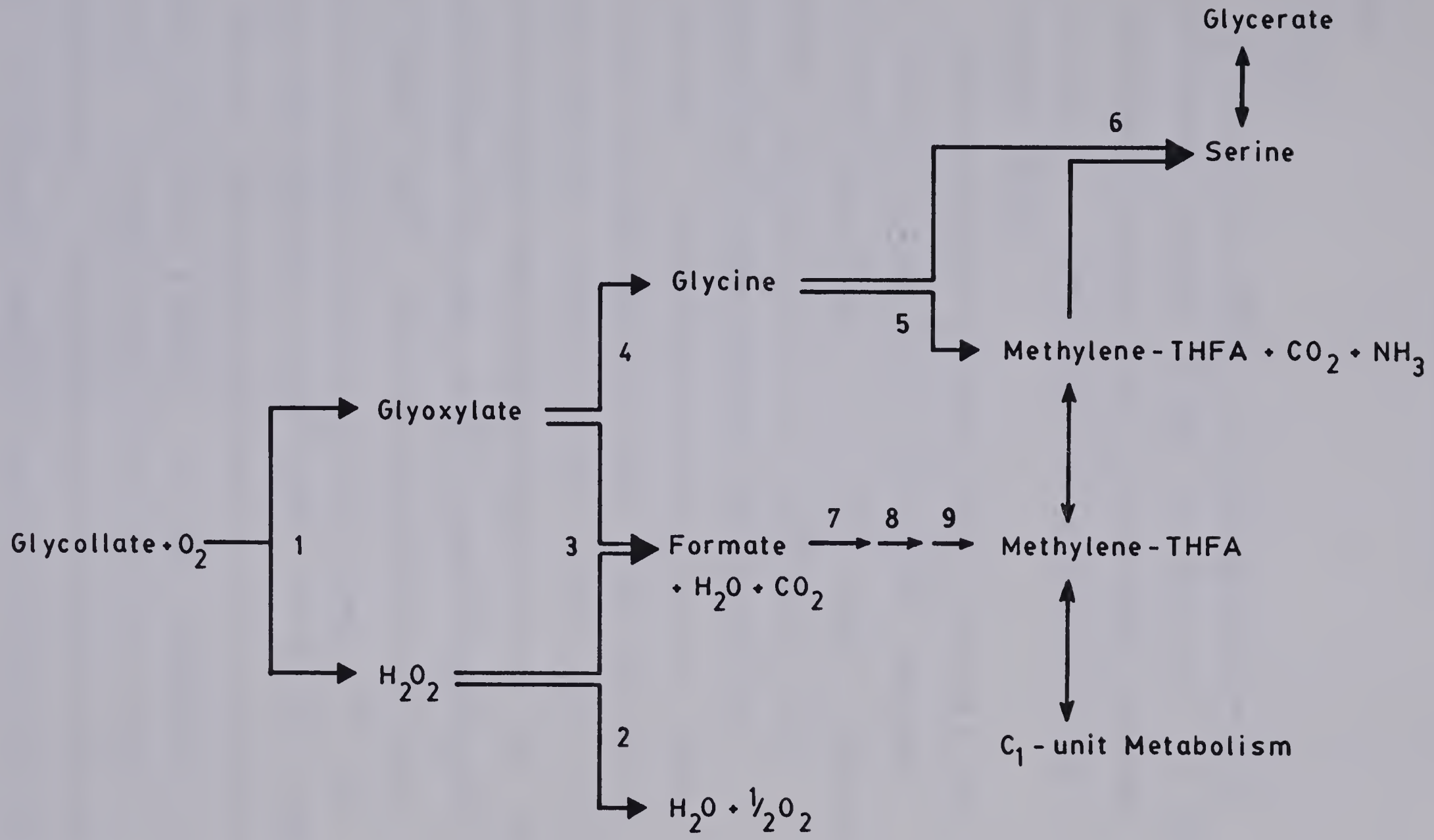
As has been discussed in the preceding sections the partial reactions of the glycollate pathway may generate C-1 units at both the formyl and methylene levels of oxidation (scheme 3). The present study has examined this possibility in greening barley leaves, and has placed particular emphasis on the generation and metabolism of formate in this system.

The greening system provides an opportunity to examine the possible interrelationships, between glycollate and folate metabolism, during a time of rapid metabolic development. That is during a period when both carbon flow through the glycollate pathway and the requirement for C-1 units in biosynthesis are increasing dramatically. In addition the greening system has the advantage of providing the opportunity to examine a large number of cells behaving in a synchronous manner. To achieve this, germination and growth is carried out initially under etiolated conditions to a stage in primary leaf development where cell division is completed. Upon illumination the cells in the mesophyll undergo development with a high degree of synchrony. This approach has been successfully exploited in a variety of other studies of higher plants (Huffaker *et al.*,1966; Chen *et al.*, 1967; Bradbeer, 1969; Tamàs *et al.*, 1970; Murray *et al.*, 1973; Hendry and Stobart, 1977a), and is now used in the present investigation.

Scheme 3. Possible interrelationships between glycollate and folate metabolism

Reaction number	Trivial Enzyme Names	Systematic Enzyme Name	E.C. Number
1	Glycollate oxidase	Glycollate:oxygen oxido-reductase	1.1.3.1
2	Catalase	Hydrogen peroxide:hydrogen peroxide oxido-reductase	1.11.1.6
3	Glycollate decarboxylation (non-enzymatic)		
4	Glycine aminotransferase	Glycine:2-oxoglutarate aminotransferase	2.6.1.4
5	Glycine synthase	5,10-Methylenetetrahydrofolate:glycine hydroxymethyltransferase	2.1.2.10
6	Serine hydroxymethyltransferase	5,10-Methylenetetrahydrofolate:ammonia hydroxymethyltransferase	2.1.2.1
7	10-Formyltetrahydrofolate synthetase or 10-HCO-H_4PteGlu synthetase	Formate:tetrahydrofolate ligase	6.3.4.3
8	5,10-Methenyltetrahydrofolate cyclohydrolase or 5,10-CH=H_4PteGlu cyclohydrolase	5,10-Methenyltetrahydrofolate 5-hydrolase	3.5.4.9
9	5,10-Methylenetetrahydrofolate dehydrogenase or 5,10-CH_2-H_4PteGlu dehydrogenase	5-Methyltetrahydrofolate:NAD^+ oxido-reductase	1.1.1.68

(Adapted from Grodzinski, 1978)


Glycerate
6
Serine
Glycine
5
4
Methylene-THFA + CO_2 + NH_3
Glyoxylate
Glycollate + O_2
1
3
Formate
7
8
9
Methylene-THFA
+ H_2O + CO_2
H_2O_2
2
H_2O + $\frac{1}{2}O_2$
C_1-unit Metabolism

MATERIALS AND METHODS

MATERIALS

Plant Material

Barley (*Hordeum vulgare* L. cv. Galt) was purchased from United Grain Growers, Edmonton, Alberta. Corn (*Zea mays* L. cv. Alta Gold), dwarf bean (*Phaseolus vulgaris* L. cv. Bountiful), and broad bean (*Vicia faba* L. cv. Broad Windsor) were purchased from Pike's Seeds, Edmonton, Alberta.

Prior to sowing all seeds were surface sterilized by soaking for 30 min in a 1% sodium hypochlorite solution, containing Tween-80, followed by a 30 min rinse in distilled water. Broad bean was germinated and grown in 6 inch pots containing sterilized loam-peat-sand (3:2:1) under greenhouse conditions (50% R.H., 22°C, 14 hr days). The plants were grown for five weeks. Barley, corn, and dwarf bean were grown from seed in 4 inch pots containing sterilized vermiculite. Growth was at 22°C and 30% R.H. in a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA). The plants were watered from below using half strength Hoagland-Epstein solution (Epstein, 1972). The plants were germinated and grown in the dark for a specific time period prior to treatment. This dark period was 6 days for barley, 13 days for corn, and 14 days for dwarf bean. After this time the etiolated plants were either illuminated or maintained in the dark as a control. This latter treatment period was up to 4 days, depending on the particular experiment. Details of the length and nature of the treatment period are given in the Results section. In the greening treatments illumination was from above and

was provided by both cool-white fluorescent lamps and incandescent bulbs. An intensity of 500 μeinsteins m^{-2} sec^{-1} was used throughout. The light intensity was measured using a LI-1905 quantum sensor (Lambda Instruments Corp., Lincoln, NE, USA). Appendix A shows an ISCO-spectroradiometer (Instrument Specialties Co. Ltd., Lincoln, NE, USA) analysis of light quality at this intensity.

Following the treatment period, typical leaves were excised and used as the experimental material. In all cases the entire leaf was used, being excised as follows:

Barley - primary leaves only, cut just above the coleoptile sheath,

Corn - primary and secondary leaves, cut at the vermiculite surface,

Dwarf bean - primary leaves, cut at the base of the petiole, and Broad bean - representative mature leaves, cut at the petiole base.

Etiolated leaves were handled in a subdued light provided by a green number 7 Kodak safelight filter and 15 watt bulb. Leaf fresh weight was determined by rapidly weighing the experimental material, in a closed lightproof container, just prior to the start of an experiment. Experiments were carried out immediately after excision.

Chemicals

α-Hydroxy-2-pyridinemethane sulphonate was purchased from Terochem Laboratories Ltd., Edmonton, Alberta. Isonicotinic acid hydrazide was purchased from the Nutritional Biochemicals Corporation, Cleveland, OH, USA. Aminopterin, dl-tetrahydrofolic acid, Sephadex-G15,

Dowex 50-X8, and Dowex 1-X8 were purchased from the Sigma Chemical Company, St. Louis, MO, USA. Other chemicals, of the highest quality commercially available, were purchased from the Fisher Scientific Company, Edmonton, Alberta, and from the Sigma Chemical Company.

[^{3}H]Formate, [^{14}C]formate, L-[3-^{14}C]serine, [1-^{14}C]glycine, [2-^{14}C]glycine, [^{3}H]toluene standard, and [^{14}C]toluene standard were purchased from the Amersham Corportion, Des Plaines, IL, USA. [2-^{14}C]-Glycollic acid was purchased from ICN Pharmaceuticals, Irvine, CA, USA.

Scintillation grade 2,5-diphenyloxazole (PPO), 1,4-di[2-(5-phenyloxazolyl)]-benzene (POPOP), and naphthalene were purchased from the Fisher Scientific Company, Koch-Light Laboratories Ltd., Colnbrook, Bucks, England, and Syndel Laboratories Ltd., Vancouver, B.C., respectively.

METHODS

Experiments involving cell-free extracts

1. Preparation of the extract

All extraction and de-salting procedures were carried out at 2°C. Leaf samples (approx. 1 g fresh wt.), were harvested, homogenized in a mortar and transferred to a centrifuge tube using a total of 6 ml of extraction buffer containing 1 g insoluble polyvinylpyrrolidone (see Loomis and Battaile, 1966). The homogenate was centrifuged at 18000 x *g* for 20 min. The pelleted residue was washed twice with 1 ml of extraction buffer and recentrifuged. The supernatants were combined and made up to 10 ml with extraction buffer. Seven ml of this extract was placed on a Sephadex

G-15 column (1x18 cm) and eluted, with the extraction buffer, in 1 ml fractions using an LKB Ultrorac 7000 Fraction collector (LKB-Produckter AB, Stockholm, Sweden). Fractions 8-17 contained the de-salted protein. De-salting generally resulted in a two fold increase in specific enzyme activity. With the exception of 5,10-CH_2-PteGlu dehydrogenase all enzyme assays were performed using the de-salted extract.

2. Enzyme assays

All assays were based on established procedures, documented in the literature. In each case, optimal activities with respect to pH, substrate and co-factor concentrations were obtained using material harvested throughout the treatment period. Both greened and etiolated material was used. Assay procedures were routinely carried out at 30°C. Buffers were adjusted to final pH at 25°C.

(a) 10-HCO-H_4PteGlu synthetase

Cell-free extracts were prepared in 0.05 M Tris (pH 8.0) containing 0.01 M 2-mercaptoethanol. The assay used was based on the method of Hiatt (1965a). The reaction system contained 100 μmoles triethanolamine buffer (pH 8.0), 150 μmoles Tris-formate (pH 8.0), 2.5 μmoles $MgCl_2$, 200 μmoles KCl, 4.0 μmoles dl-H_4PteGlu in 0.05 M 2-mercaptoethanol, 2.5 μmoles ATP and cell-free extract (*ca.* 0.3 mg protein), in a total volume of 1.5 ml. The controls consisted of complete systems lacking the ATP.

After a 5 min pre-incubation the reaction was initiated by addition of ATP, and the mixture was incubated for a further 10 min. The reaction was stopped by adding 1.5 ml 1M HCl and the reaction mixture allowed to stand for 10 min at 30°C. Denatured protein was removed by centrifugation. Under these conditions, the 10-HCO-H_4PteGlu

formed in this reaction was converted to 5,10-CH=H_4PteGlu. The latter compound was estimated spectrophotometrically using a Beckman DB-G recording spectrophotometer (Beckman Instruments Inc., Palo Alto, CA, USA) at 350 nm, using an E_{max} value of 24,900 (Himes *et al.*, 1962).

One unit of the enzyme was taken as the amount which produced 1 nmole of product per min per 3 ml final volume.

(b) 5,10-CH_2-H_4PteGlu dehydrogenase

The extraction buffer for this enzyme was 0.01 M potassium phosphate (pH 7.5) containing 0.01 M 2-mercaptoethanol and 20% v/v glycerol. Activity was assayed spectrophotometrically (Wong and Cossins, 1966). The reaction system contained 150 µmoles potassium phosphate (pH 7.5), 3.5 µmoles formaldehyde and 1 µmole dl-H_4PteGlu in 0.05 M 2-mercaptoethanol. This mixture was preincubated for 4 min, to allow formation of 5,10-CH_2-H_4PteGlu. The enzyme preparation (*ca.* 0.3 mg protein) was then added, followed by a further incubation of 2 min. The reaction was initiated by the addition of 0.5 µmoles NADP. A complete reaction system lacking the NADP served as a control. The reaction was stopped after 2 min by adding 1.0 ml of M HCl and left for 10 min at 30°C to optimize formation of the product, 5,10-CH=H_4PteGlu. Denatured protein was removed by centrifugation and absorbance at 350 nm was measured. The E_{max} = 24,900 value of Himes and Rabinowitz, (1962) was used to calculate product formed. One unit of the enzyme was taken as the amount producing 1 nmole of product per min per 3 ml final volume.

(c) Serine hydroxymethyltransferase

The enzyme was extracted using 5 mM potassium phosphate (pH 6.9) containing 5 mM 2-mercaptoethanol. The assay method of

Taylor and Weissbach (1965) was employed. Radioactivity in 5,10-CH_2-H_4PteGlu, produced in the reaction, was trapped with carrier formaldehyde and converted to the dimedon addition product. The reaction system contained 30 µmoles potassium phosphate (pH 8.0), 1 µmole dl-H_4PteGlu in 0.05 M 2-mercaptoethanol, 0.1 µmole pyridoxal-5'-phosphate, 0.1 µCi of L-[3-^{14}C]serine (54 µCi/µmole) and cell-free extract (*ca.* 0.2 mg protein) in a total of 0.7 ml. The control comprised a complete system minus the plant extract. All components were preincubated for 5 min. The reaction was initiated by adding L-[3-^{14}C]serine and was terminated 15 min later by adding in succession, 0.3 ml of 1.0 M sodium acetate (pH 4.5), 0.2 ml of 0.1 M formaldehyde and 0.3 ml of 0.4 M dimedon (in 50% v/v ethanol). The formation of the HCHO-dimedon derivative was achieved by heating the reaction mixture in a boiling water bath for 5 min. After cooling for 5 min in an ice bath the dimedone adduct was extracted by vigorous shaking with 3 ml toluene at room temperature. The aqueous and toluene phases were separated by centrifugation. The toluene phase was removed for measurement of ^{14}C.

(d) Glycollate oxidase

The extraction buffer used to prepare the cell-free extract for this assay was 0.05 M Tris (pH 8.0) containing 0.01 M 2-mercaptoethanol. The enzyme was assayed spectrophotometrically by monitoring the rate of increase in absorbance at 324 nm due to the formation of glyoxylic acid phenylhydrazone (Murray *et al.*, 1973). The reaction system contained 200 µmoles Tris-chloride (pH 7.8), 20 µmoles glycollic acid, 10 µmoles phenylhydrazine HCl (pH 7.0), and cell-free extract (*ca.* 0.3 mg protein) in a total volume of 3 ml. The control consisted of a complete system minus the glycollate. After a 3 min pre-incubation,

the reaction was initiated by addition of the substrate. At 30°C there was a 1 min lag in product formation after which absorbance increased linearly for up to 10 min. The lag could be overcome by adding 0.05 μmoles FMN before the addition of glycollate. However, as FMN was found to be inhibitory in some cases it was routinely omitted. A standard curve was prepared using known amounts of glyoxylate.

One unit of the enzyme was taken as the amount which produced 1 nmole of product per min per 3 ml final volume.

(e) Glyoxylate decarboxylation

Cell-free extracts were prepared in 0.02 M glycylglycine (pH 7.5), and assayed by the method of Halliwell and Butt (1974). In this method glyoxylate is decarboxylated to formate and CO_2. The formate is then chemically reduced to formaldehyde which is estimated spectrophotometrically by the method of Nash (1953). The reaction system contained 99 μmoles glycylglycine (pH 8.0), 0.09 μmoles FMN, 45 μmoles sodium glyoxylate, and cell-free extract (*ca.* 0.3 mg protein) in a final volume of 3 ml. The reaction was initiated by adding the extract and was terminated after 90 min by pipetting 1 ml of the mixture into a tube containing 0.25 ml 12 M HCl placed in an ice bath. Magnesium ribbon (60 mg) was added followed by dropwise additions of 12 M HCl, to a final total HCl volume of 1 ml, over a 10 min period. Under these conditions the formate produced from glyoxylate decarboxylation was quantitatively reduced to formaldehyde (Wood and Gest, 1957). The reaction mixture was neutralized by adding 3 ml 1 M cold NaOH, and the precipitated $Mg(OH)_2$ removed by centrifugation. A 2 ml aliquot of the supernatant was added to 2 ml of Nash's reagent B (2 ml redistilled acetylacetone plus 3 ml glacial

acetic acid plus 150 g ammonium acetate made up to 1 litre with distilled water). This mixture was incubated at 37°C for 45 min. Diacetyldihydrolutidine, the reaction product, was measured spectrophotometrically at 412 nm. The control consisted of a complete reaction system minus the plant extract. In the control, the extract was only added after 90 min incubation period and 1 ml was immediately withdrawn into a tube containing 0.25 ml 12 M HCl. No enzymic glyoxylate decarboxylation occurred under these conditions. The control was then treated in exactly the same way as a complete system. Controls of this type allowed two factors to be corrected for, firstly, possible non-enzymic glyoxylate decarboxylation, and secondly, possible inhibition of colour development by the cell-free extract.

A standard curve was prepared using varying amounts of formate in a complete reaction system. Here the procedure used was the same as that described above for the control. The control for the standard curve contained no added formate and was also treated in the same manner as for the control above.

One unit of the enzyme was taken as the amount which produced 1 nmole of product per min per final reaction volume.

3. Estimation of protein

Protein content of cell-free extracts was determined colorimetrically using the method of Lowry *et al.* (1951). Crystalline egg albumin was used as a reference standard. All determinations were made in triplicate.

4. Estimation of chlorophyll

The chlorophyll content of leaf tissue was determined colorimetrically using the method described by Harborne (1973). All

determinations were made in triplicate using 80% v/v acetone extracts.

Radioisotope feeding experiments

1. General procedure

After each treatment period three representative leaves were excised as previously described. The leaves were cut across the lamina into 2 mm sections. The sections were quickly washed with a small quantity (*ca.* 20 ml) of distilled water and placed in a Warburg flask containing 2.8 ml half strength Hoagland-Epstein solution adjusted to pH 7.5 with 1 M NaOH. The flask was attached to a manometer and the closed system placed in an illuminated Warburg apparatus (Bronwill Scientific Inc., Rochester, NY, USA). Illumination was provided by incandescent bulbs with a mean light intensity at the base of the flask, of 500 μeinsteins m^{-2} sec^{-1}. All radioisotope feeding experiments were carried out at 25°C, with constant shaking. Pre-incubation and feeding periods depended upon the particular experiment, and in some cases the flasks were kept in the dark throughout. More information on these conditions appears in the Results section. Labelled compounds were used without dilution of initial specific radioactivity, distilled water being used to dilute or dissolve the material from the supplier. In all experiments the desired concentration and dpm were contained in a 0.2 ml aliquot.

In a typical feeding experiment the flask and contents were first pre-incubated, the radioisotope was introduced, and the flask further incubated for the feeding period. At the end of this period the contents of the flask were quickly transferred to a chilled mortar containing ice-cold ethanol (95% v/v) and *ca.* 1 g of fine sea sand.

The leaf sections were thoroughly homogenized at 2°C. The homogenate was quantitatively transferred to a centrifuge tube using ice-cold ethanol. After centrifugation at 18000 x *g* for 10 min the residue was washed 3 times with 10 ml aliquots of water and recentrifuged. The insoluble residue was retained for analysis of protein amino acids. The combined supernatants were dried *in vacuo* at 40°C on a Buchler flash-evaporator (Buchler Instruments, Fort Lee, NY, USA). In the case of [^{3}H]- or [^{14}C]formate feeding residual formate was removed by sequentially washing and redrying the residue with 20 ml of 8 M formic acid, 4 M formic acid, and distilled water. After evaporation to dryness the residue was extracted with three 5 ml aliquots of diethyl ether, to remove ether solubles. The volume of the ether extract was measured and its radioactivity determined using liquid scintillation counting (see section 4). The residue was redissolved in a total of 15 ml distilled water and fractionated into amino acid, organic acid and sugar fractions as described below (section 3).

2. Acid hydrolysis of the insoluble residue

The samples of the insoluble residues, obtained above, were dried with acetone and suspended in 10 ml of 6 M HCl. After degassing, the samples were hydrolysed in a sealed tube by autoclaving at 110°C for 16 hr (Beckman Instruments, 1970). The hydrolysate was filtered and dried *in vacuo* at 40°C. The residue was redissolved in 10 ml distilled water and redried. This process was repeated until the hydrolysate was acid-free. The hydrolysate was then dissolved in 10 ml distilled water, and the protein amino acids present recovered by ion exchange chromatography as described below.

3. Ion exchange chromatography

The amino acids were separated from the organic acids and sugars by passing the water soluble material, obtained above, through a 8x1 cm column of Dowex 50W-X8 (hydrogen form). The organic acids and sugars passed through this column in water, and were separated from each other using a 8x1 cm column of Dowex 1-X8 (formate form) according to the procedure of Canvin and Beevers (1961). The sugars passed through the column in water and the organic acids were eluted using successive washings of 40 ml 4 M formic acid and 30 ml 8 M formic acid. The separated sugar and organic acid fractions were dried *in vacuo* at 40°C. The residues were redissolved in 5 ml distilled water and their radioactivities determined as described below. The amino acids were eluted from the Dowex 50W-X8 column using 50 ml 6 M HCl. The amino acid fraction was dried *in vacuo* at 40°C, and finally redissolved in 3 ml 0.2 M sodium citrate buffer (pH 2.2). Radioactivity was determined prior to amino acid analysis.

Drying the amino acid eluate under vacuum at 40°C resulted in the partial hydrolysis of asparagine and glutamine. Approximately 10% of the free asparagine and 20% of the free glutamine was hydrolysed to aspartic and glutamic acids, respectively.

4. Counting of radioactive samples

Radioactivity was measured using liquid scintillation counters (Isocap/300 model, Nuclear Chicago Corp. IL, USA and a Mark III, 6881 liquid scintillation system, Tracor Analytic, Elkgrove Village, IL, USA). Aliquots (0.1-0.5 ml) of the labelled samples were counted in 10 ml Bray's solution, 4 g 2,5-diphenyloxazol (PPO) and 0.2 g 1,4 di[2-(5-phenyloxazolyl)]benzene (POPOP) plus 60 g

naphthalene, 100 ml methanol, 20 ml ethyleneglycol and dimethoxyethane to make 1 litre (Bray, 1960). To facilitate dpm conversion, counting efficiencies were determined by the sample channels ratio (Bush, 1963) and external standard channels ratio (Higashimura *et al.*, 1962) methods.

Quenched standards were prepared using [^{3}H]- and [^{14}C]toluene standards. All counts were corrected for background (*ca.* 25 cpm) and were only regarded as significant if at least twice this level.

Analysis of amino acid pools

The level of individual free amino acids in the plant extracts was determined using a Beckman Automatic Amino Acid Analyser, model 121 (Clandinin and Cossins, 1972). Whole leaves were excised and homogenized with ice-cold ethanol in a mortar at 0°C. After centrifugation at 18000 x *g* for 10 min, the insoluble residue was washed three times with 10 ml distilled water and recentrifuged. The combined supernatants were dried *in vacuo* at 40°C and the residue redissolved in 15 ml distilled water. The free amino acids present were recovered by ion-exchange chromatography (see section 3). The amino acid fraction was dried *in vacuo* at 40°C and the residue redissolved in 3.0 ml of 0.2 M sodium citrate buffer (pH 2.2). Aliquots of this solution were analysed using PA28 spherical resin (Beckman Instruments Inc., CA, USA). The eluting buffer for separating the neutral and acidic amino acids was 0.20 M citrate at pH 3.22 and 4.25. The basic amino acids were eluted from PA35 resin using 0.35 M citrate buffer (pH 5.25). The pH of all buffers were measured at 22°C and elution was carried out at 53°C, with a flow rate of 70 ml/hr.

Eluted amino acids were reacted with ninhydrin at 100°C. The system was calibrated using authentic amino acid standards of known concentration.

In general, amino acids were eluted as discrete peaks, however asparagine, serine and glutamine co-chromatographed. To obtain accurate serine levels, asparagine and glutamine were removed by hydrolysing them to aspartate and glutamate, respectively. Complete hydrolysis was achieved by refluxing 1 ml of the amino acid solution (see above) for 1 hr with 10 ml 8 M HCl. After hydrolysis the solution was dried *in vacuo* at 40°C, the the hydrolysate redissolved in 3 ml 0.2 M sodium citrate buffer (pH 2.2), prior to amino acid analysis.

For determination of individual radioactive amino acids aliquots of the citrate buffer solution were subjected to stream dividion amino acid analysis. In this technique half of the sample was used for quantitative amino acid determination by the ninhydrin reaction, and the other half of the sample was collected in 2 ml fractions using a fraction collector. Radioactivity of these fractions was determined via the liquid scintillation counter. The identity of labelled amino acids was confirmed by co-chromatographing labelled peak areas with authentic amino acids, using the ninhydrin reaction.

Inhibitor studies

(a) α-Hydroxy-2-pyridinemethane sulphonate (αHPMS)

Barley seeds were germinated and grown in the dark for 7 days. Etiolated leaves were cut at the surface of the vermiculite and re-cut under water 10 cm from the leaf tip, to preserve xylem

integrity. The cut ends were placed in beakers containing 20 ml of αHPMS solution (up to 10^{-2} M αHPMS in half strength Hoagland-Epstein solution, pH 7.5). The leaves were supported vertically by means of a wire mesh placed over the mouth of the beaker. The leaves were illuminated for 24 hr at 500 μeinsteins m^{-2} sec^{-1}, in a growth chamber. After illumination the leaves were removed and used in either feeding experiments or for the determination of enzyme activities.

(b) Isonicotinic acid hydrazide (INH)

Barley seeds were germinated and grown in the dark for 6 days. The etiolated leaves were illuminated at 500 μeinsteins m^{-2} sec^{-1}, and growth was continued for two more days. After this period the greened leaves were harvested and placed in Warburg flasks prior to radioactive formate feeding, as previously described. INH solution, 2.8 ml (up to 0.36 M INH in half strength Hoagland-Epstein solution, pH 7.5) was added to the flasks which were pre-incubated for 60 min at 25°C and 500 μeinsteins m^{-2} sec^{-1} in an illuminated Warburg apparatus. After the pre-incubation period, 0.2 ml [^{3}H]- or [^{14}C]formate was added and the flasks incubated a further 20 min in the light. At the end of this feeding period the flask contents were rapidly homogenized at 2°C in a mortar containing ice-cold ethanol and sea sand. The homogenate was fractionated as described above.

(c) Aminopterin

Barley seeds were germinated and grown in the dark for 6 days. Three leaves were excised just above the coleoptile sheath, cut into 2 mm sections and placed in a Warburg flask containing 2.8 ml aminopterin solution (up to 10^{-3} M aminopterin in half strength Hoagland-Epstein solution, pH 7.5). The flask was placed on a Warburg

apparatus and the leaf segments were incubated for 24 hr with illumination at 500 μeinsteins m^{-2} sec^{-1}. After this greening period [^{3}H]- or [^{14}C]formate in 0.2 ml was fed to the segments for 20 min in the light. At the end of this period the flask contents were rapidly homogenized at 2°C in a mortar containing ice-cold ethanol and sea sand. The homogenate was analyzed as described above in earlier sections.

RESULTS

Preliminary studies of the greening barley leaf

The majority of studies leading to this thesis were carried out on *Hordeum vulgare* L. cv. Galt. Previous studies of barley (Huffaker *et al.*, 1966; Lloyd, 1976) had indicated that light intensity plays an important role in leaf development during greening. In particular, these studies showed direct correlations between light intensity and enzyme activities; in addition optimal light intensities for the enzymes studied were noted. As the present study was aimed at examining relationships between C-1 metabolism and associated metabolic pathways in the greening barley leaf, it was felt that these could best be demonstrated under conditions of optimal greening. To establish this, six-day etiolated leaves were greened for 48 hrs at various light intensities prior to harvesting (Materials and Methods). Three parameters were examined; firstly chlorophyll content as an indicator of both chloroplast and photosynthetic development, secondly soluble protein for overall cellular metabolic development, and thirdly glycollate oxidase because of its role in the glycollate pathway. The results are summarized in Figure 1. From these data 500 μeinsteins $m^{-2}sec^{-1}$ was chosen as optimal, and this light intensity was used in all subsequent studies.

As noted in the Introduction, the activity of certain photosynthetic enzymes increases during greening. Studies of barley (Huffaker *et al.*, 1966; Tamàs *et al.*, 1970; Lloyd, 1976) have shown that in many cases the first 2 days of greening represent the period of maximum change. Since the present study required such a period it was necessary to determine when it occurred. To accomplish this, six-day etiolated leaves were either

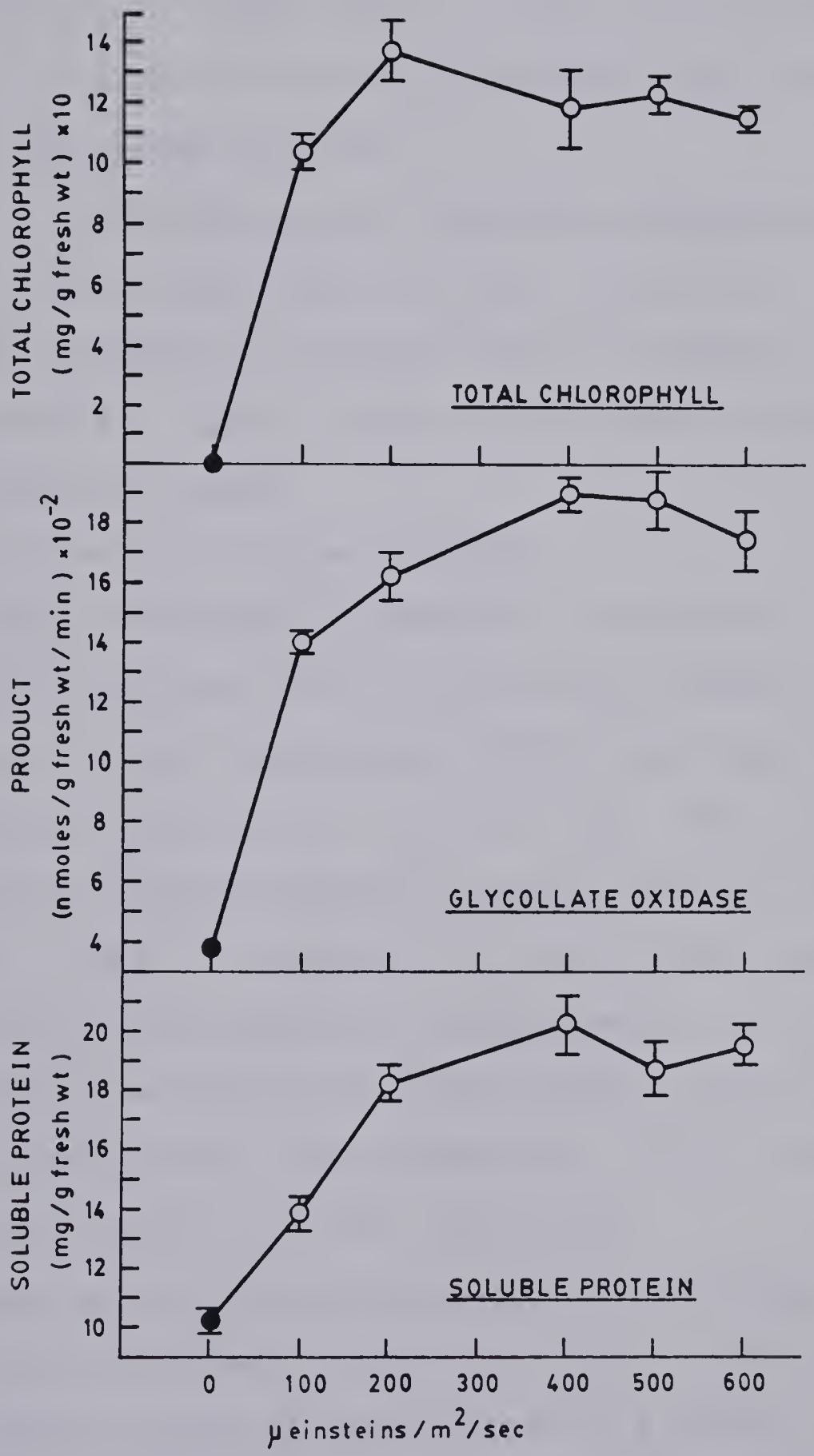
FIGURE 1

EFFECT OF LIGHT INTENSITY ON CHLOROPHYLL, GLYCOLLATE OXIDASE, AND SOLUBLE PROTEIN DURING GREENING

All procedures were as outlined in the Materials and Methods. Six-day old etiolated leaves were greened at different light intensities for two days. The treated leaves were then harvested and assayed. Each data point represents a mean value ± S.E.M., obtained from triplicate assays performed on each of three separate experiments.

—○— treatments involving greening

—●— treatment maintaining etiolated conditions


TOTAL CHLOROPHYLL
(mg/g fresh wt) ×10
14
12
10
8
6
4
2
TOTAL CHLOROPHYLL
PRODUCT
(n moles/g fresh wt/min) ×10⁻²
18
16
14
12
10
8
6
4
GLYCOLLATE OXIDASE
SOLUBLE PROTEIN
(mg/g fresh wt)
20
18
16
14
12
10
SOLUBLE PROTEIN
0
100
200
300
400
500
600
μ einsteins/m²/sec

greened or held in the dark as controls, for up to 5 days. The treated leaves were subsequently harvested and assayed for chlorophyll, glycollate oxidase, soluble protein, and soluble amino acids (Materials and Methods). The data (Figure 2) clearly show that the first 2 days of greening represent a dynamic period with maximal changes occuring in the parameters examined. Consequently the majority of subsequent experiments were carried out on tissues obtained during this period.

It is interesting to note that while other parameters increased, the soluble amino acid pool declined in the greening leaves. This trend was reflected by the majority of individual amino acids detected, including serine (Appendix B). Glycine, however did not show an appreciable change in pool size during this period.

The effect of greening on enzyme activities

Enzymes which could be involved in the generation, from glycollate, of C-1 units at the formyl level of oxidation were examined in greening barley leaves. In this study six-day etiolated leaves were either greened or held in the dark as controls, for up to 2 days. Samples were taken throughout this period and assayed for various enzyme activities (Materials and Methods). Due to large increases in soluble protein during the greening period (Figure 2) enzyme activities are expressed on a fresh weight basis, and are therefore in keeping with previous studies (Huffaker *et al.*, 1966; Chen *et al.*, 1967; Bradbeer, 1968; Graham *et al.*, 1970; Feierabend and Beevers, 1972a; Murray *et al.*, 1973; Lloyd, 1976).

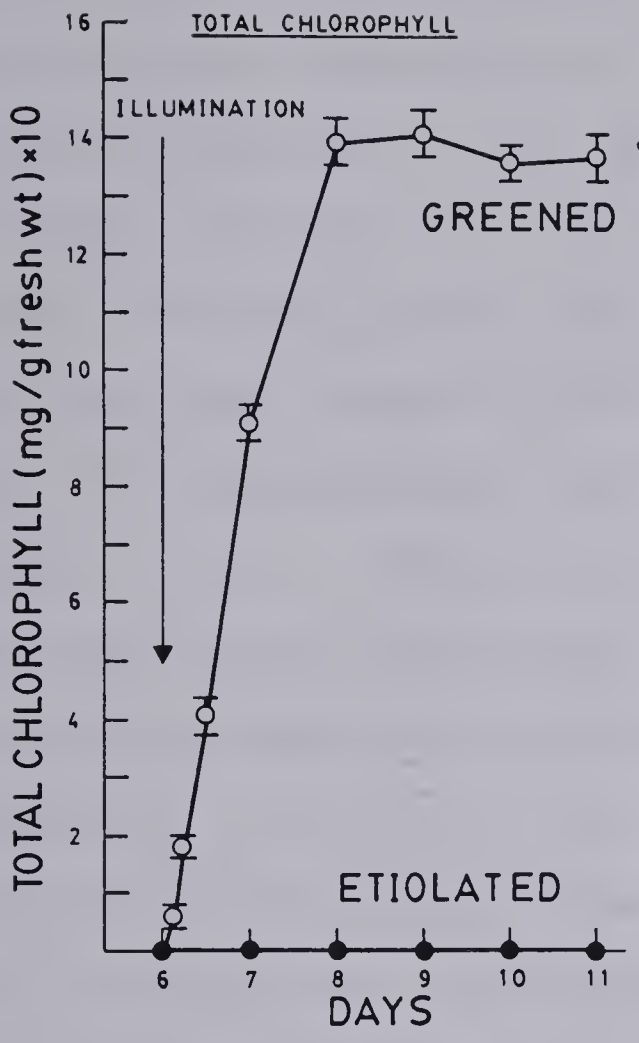
1. Glyoxylate decarboxylation and the generation of formate

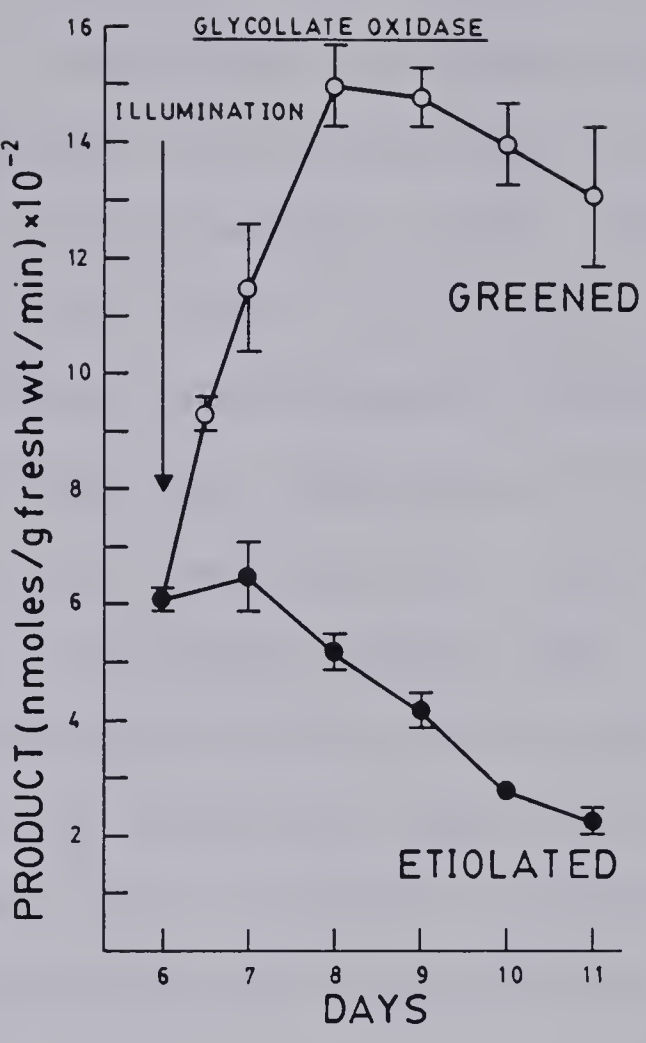
The rapid increase in glycollate oxidase activity during the first 2 days of greening (Figure 2) clearly represents a potential increase in the glyoxylate pool. Halliwell and Butt (1974) have shown that peroxisomes of spinach and spinach beet leaves readily catalyse the decarboxylation of

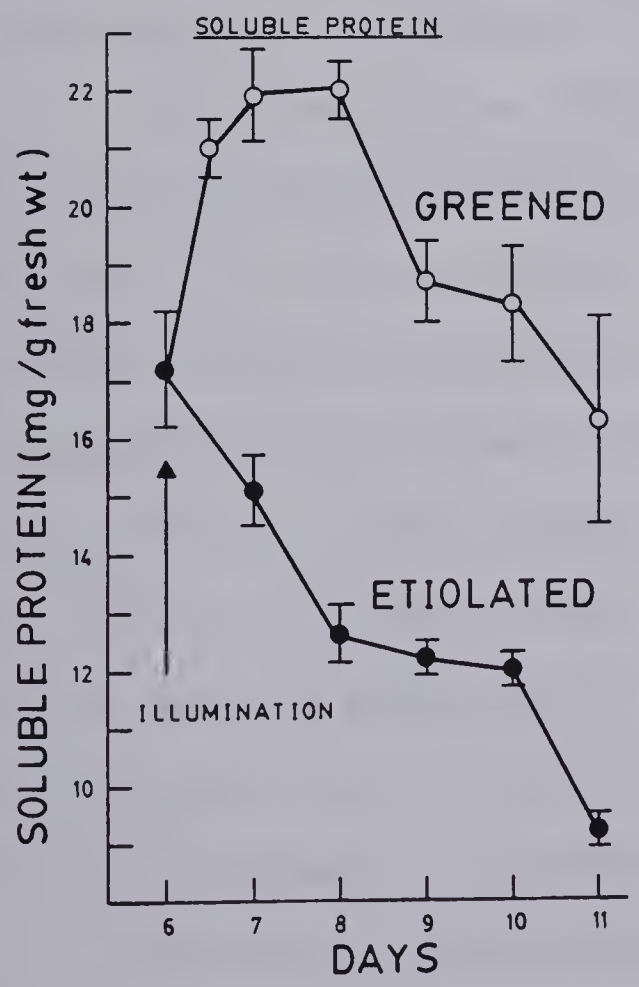
FIGURE 2

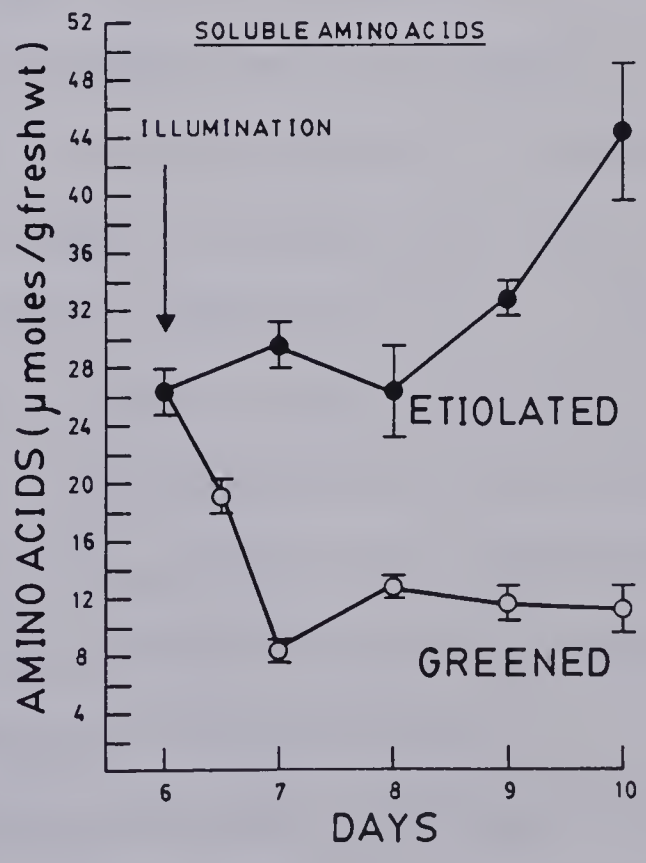
CHANGES IN CHLOROPHYLL, GLYCOLLATE OXIDASE, SOLUBLE PROTEIN, AND THE TOTAL AMINO ACID POOL DURING GREENING, AT OPTIMUM LIGHT INTENSITY

Procedures followed were those outlined in the Materials and Methods. Six-day old etiolated leaves were either greened at 500 μeinsteins m^{-2} sec^{-1}, or held in the dark as controls. Samples were taken during a subsequent five day period and assays carried out. With the exception of the soluble amino acid pool data, each data point represents a mean value ± S.E.M., obtained from triplicate assays performed on each of three separate experiments. The soluble amino acid data was obtained by summing individual amino acid values. These values are presented in Appendix B.


TOTAL CHLOROPHYLL
ILLUMINATION
GREENED
ETIOLATED
TOTAL CHLOROPHYLL (mg/g fresh wt) ×10
DAYS


GLYCOLLATE OXIDASE
ILLUMINATION
GREENED
ETIOLATED
PRODUCT (nmoles/g fresh wt/min) ×10⁻²
DAYS


SOLUBLE PROTEIN
GREENED
ETIOLATED
ILLUMINATION
SOLUBLE PROTEIN (mg/g fresh wt)
DAYS


SOLUBLE AMINO ACIDS
ILLUMINATION
ETIOLATED
GREENED
AMINO ACIDS (μ moles/g fresh wt)
DAYS

glyoxylate to formate and CO_2. Barley leaf extracts were examined for this ability during the greening period (Figure 3). After correction for non-enzymic glyoxylate decarboxylation (see Materials and Methods) formate production was demonstrated in both greened and etiolated leaves. This reaction was enzymic since boiled extracts failed to generate formate. The data (Figure 3) show that formate production increased as both greened and etiolated leaves aged. However significantly higher rates were obtained for leaves which had been greened for 1 to 2 days. The apparent Michaelis constants (Table 1), obtained throughout the study period, revealed no significant change in the affinity for either glycollate or FMN. Consequently the observed changes can be considered due to an increase in enzyme activity. The parallels in the data for these two enzymes also imply that the sequence from glycollate→glyoxylate→formate+CO_2 may be operative in greening barley leaves, and that carbon flow in this pathway might increase rapidly during the first 2 days of greening.

2. Folate enzymes, and the activition of formate as a C-1 unit

As outlined in the Introduction, many organisms activate formate via the 10-formyltetrahydrofolate synthetase reaction (Scheme 2, reaction 3). The product, 10-formyltetrahydrofolate, can be reduced to the methylene level of oxidation through the combined action of cyclohydrolase and 5,10-methylene-tetrahydrofolate dehydrogenase (Scheme 2, reactions 4 and 5). Although there is good evidence for these enzymes in plants (Hiatt, 1965a; Iwai *et al.*, 1967; Cossins *et al.*, 1970) there is no information on their possible activation or synthesis during greening. The present study examined 10-formyl-tetrahydrofolate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase in relation to greening, as summarized in Figures 4 and 5.

Both enzymes were detected in etiolated and greened leaves. In each case, the apparent Michaelis constants (Tables 2 and 3) did not change

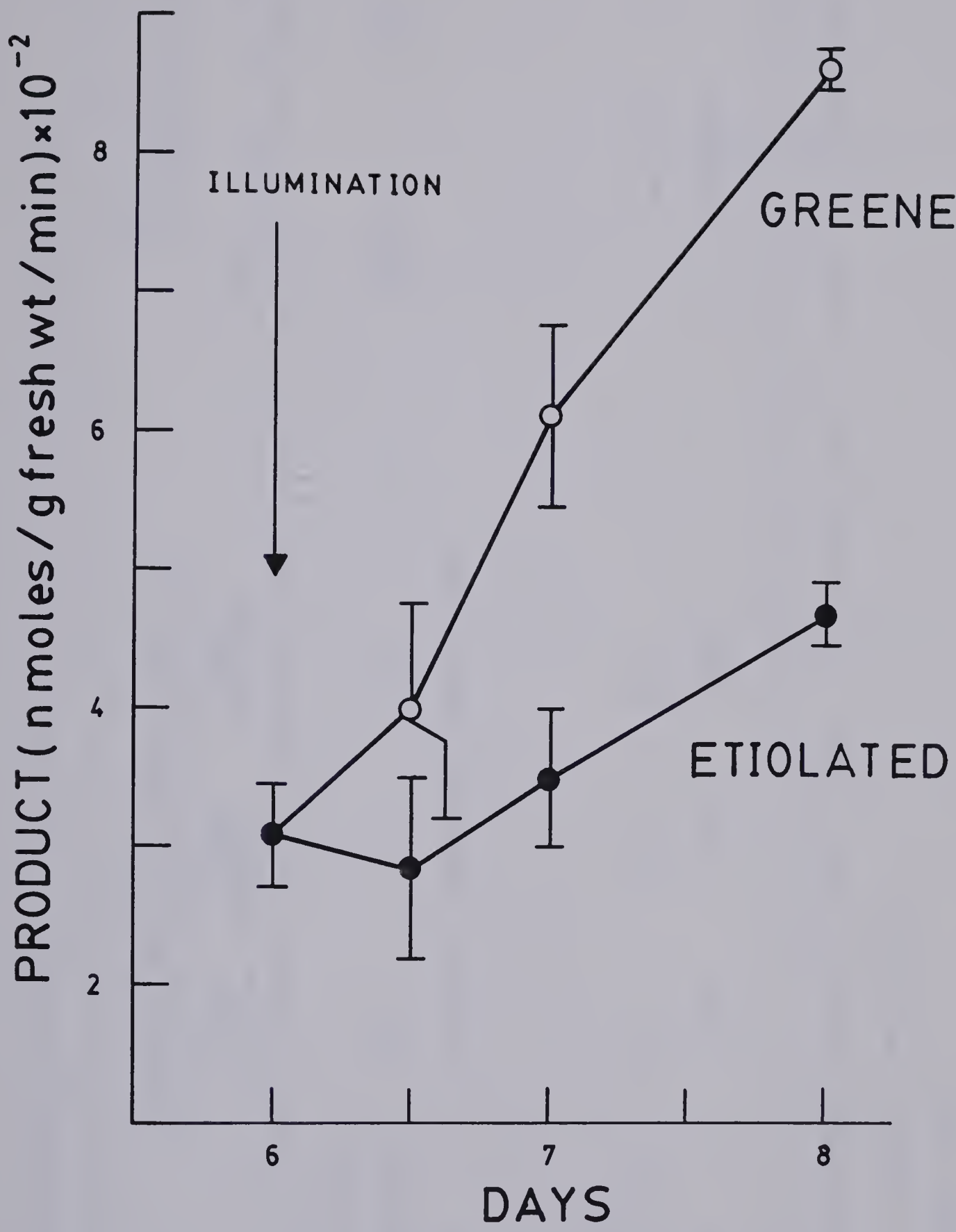


FIGURE 3. FORMATE PRODUCTION FROM GLYOXYLATE DURING GREENING

The complete reaction system (final pH 8.0) and assay procedures are described in the Materials and Methods. Each data point represents a mean value ± S.E.M., obtained from triplicate assays performed on each of three separate experiments. Specific activities (nmoles product/min/mg protein) were as follows: six-day etiolated 32.6 ± 2.9, eight-day greened 70.9 ± 2.3, eight-day etiolated 65.7 ± 2.0.

TABLE 1. Apparent Michaelis constants of glycollate oxidase and glyoxylate decarboyxlation.

	Km values for barley leaf extracts (mM)			Published Km values	
	6 day etiolated	8 day greened	8 day etiolated	Plant source	Km (mM)
1. Glycollate oxidase					
glycollate	0.4	0.3	0.4	spinach	0.4[a]
FMN	no specific requirement demonstrated				
2. Glyoxylate decarboxylation					
glyoxylate	27.0	61.0	29.0	no data	
FMN	0.002	0.004	0.005	no data	

Km values were obtained on de-salted leaf extracts. This procedure, and the complete reaction systems are described in the Materials and Methods.

[a] Zelitch and Ochoa, 1953.

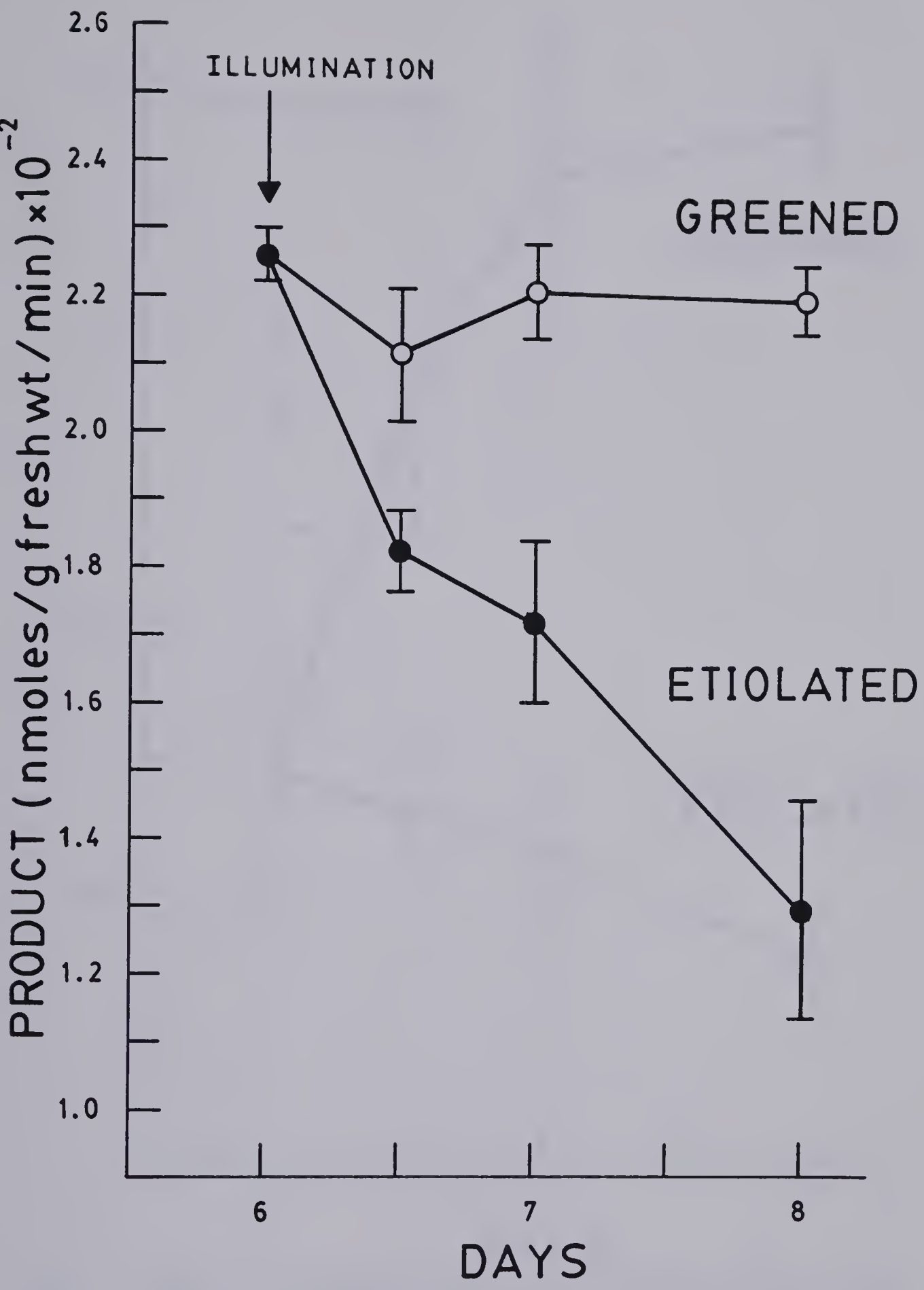


FIGURE 4. ACTIVITY OF 10-FORMYLTETRAHYDROFOLATE SYNTHETASE DURING GREENING

The complete reaction system (final pH 8.0) and assay procedures are described in the Materials and Methods. Each data point represents a mean value ± S.E.M., obtained from triplicate assays performed on each of three separate experiments. Specific activities (nmoles product/min/mg protein) were as follows: six-day etiolated 20.5 ± 3.1, eight-day greened 13.3 ± 3.2, eight-day etiolated 14.6 ± 2.0.

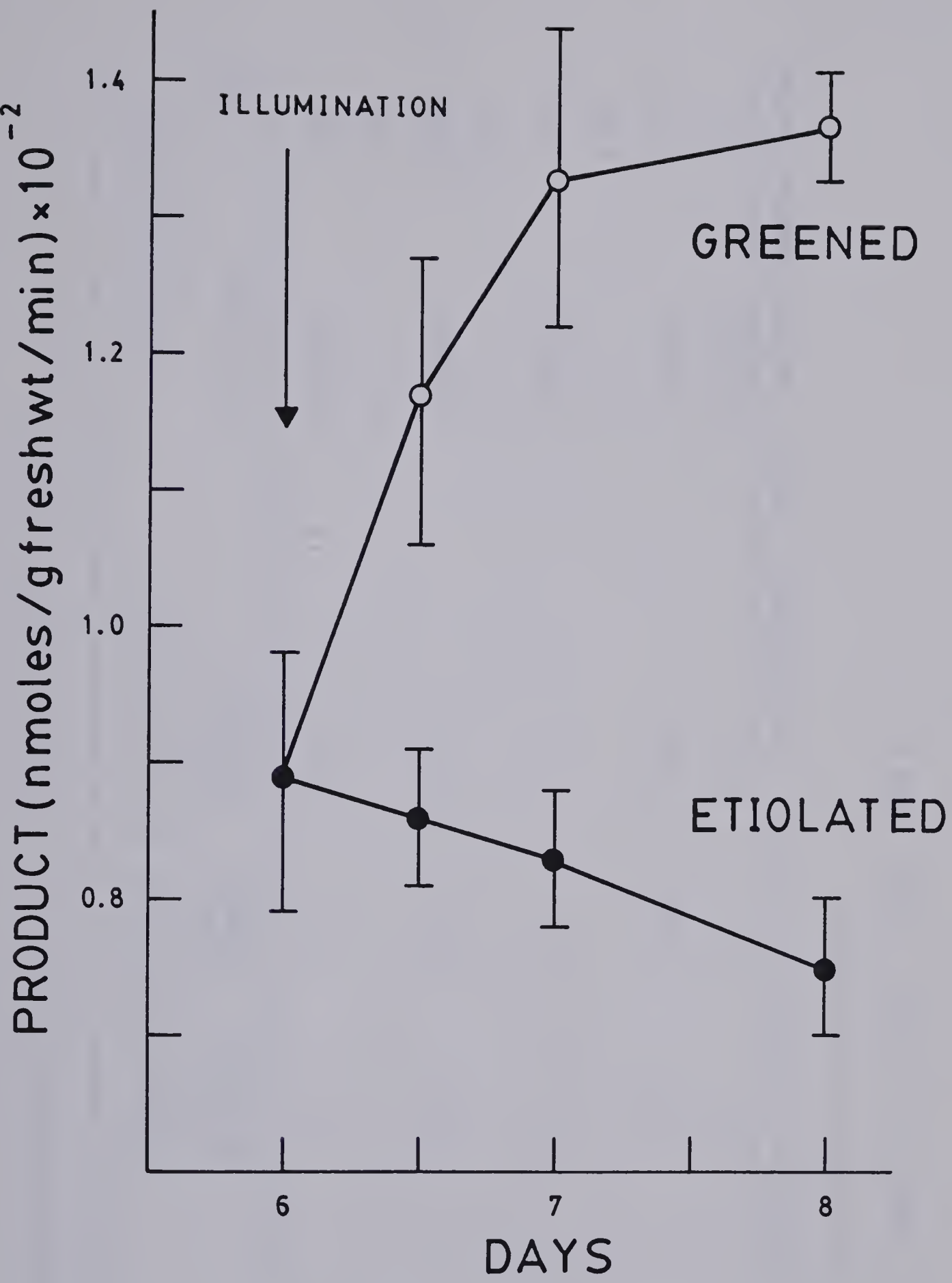


FIGURE 5. ACTIVITY OF 5,10-METHYLENETETRAHYDROFOLATE DEHYDROGENASE DURING GREENING

The complete reaction system (final pH 7.5) and assay procedures are described in the Materials and Methods. Each data point represents a mean value ± S.E.M., obtained from triplicate assays performed on each of three separate experiments. Specific activities (nmoles product/min/mg/protein) were as follows: six-day etiolated 5.7 ± 0.5, eight-day greened 6.8 ± 0.6, eight-day etiolated 6.3 ± 0.9.

Table 2. 10-Formyltetrahydrofolate synthetase : apparent Michaelis constants

	Km values for barley leaf extracts (mM)			Published Km values	
	6 day etiolated	8 day greened	8 day etiolated	Plant source	Km (mM)
THFA	0.7	0.4	0.5	Spinach	0.9[a]
				Pea	5.6[b]
Formate	2.9	2.8	3.3	Spinach	33.0[a]
				Pea	21.0[b]
ATP	0.2	0.2	0.3	Spinach	0.1[a]
				Pea	0.5[b]
Mg^{++}	0.007	0.005	0.006	Spinach	0.1[c]
K^{+}	0.5	0.2	0.3	Spinach	13.0[c]

Km values were obtained on de-salted leaf extracts. This procedure, and the complete reaction system are described in the Materials and Methods.

[a] Hiatt, 1965 a; [b] Iwai *et al.*, 1967; [c] Hiatt, 1965 b.

Table 3. 5,10-Methylenetetrahydrofolate dehydrogenase : apparent Michaelis constants

	Km values for barley leaf extracts (mM)			Published Km values	
	6 day etiolated	8 day greened	8 day etiolated	Plant source	Km (mM)
THFA	0.002	0.004	0.002	Pea	0.2 [a]
Formaldehyde	0.1	0.2	0.3	Pea	0.8 [a]
NADP	0.03	0.01	0.05	Pea	0.0004 [a]

Km values were obtained on de-salted leaf extracts. This procedure and the complete reaction system are described in the Materials and Methods.

[a] Cossins *et al.*, 1970

during the study period, showing that no changes occurred in the affinity for either substrates or co-factors. In greening leaves synthetase activity was unchanged, but was significantly higher than that of the etiolated controls (Figure 4). In contrast, the dehydrogenase showed a rapid increase in activity in response to greening (Figure 5). These data also show that a greater potential for the activation of formate, and subsequent reduction of the active C-1 unit exists in extracts of greening barley leaves. In addition, parallels between the rates of activity of these enzymes, and those of formate synthesis, suggest a potential *in vivo* flow from formate→10formyl-H_4PteGlu→5, 10-methyleneH_4PteGlu in the barley leaf, which appears to increase rapidly during the first 2 days of greening.

The incorporation of radioactive formate by barley leaves

In a preliminary study, the activation of formate *in vivo*, and the subsequent metabolism of the active C-1 unit was examined using eight-day old barley leaves, which had either been greened or held in the dark as controls. This procedure and the subsequent fractionations and analyses have been described (Materials and Methods). A time course for ^{3}H incorporation into the amino acid fraction is shown in Figure 6. These data indicate that optimal ^{3}H labelling occurred after a 20 min feeding period. As this period also resulted in maximal labelling of individual amino acids, it was used in all subsequent experiments.

Since many of the enzymes involved in the pathways of photosynthesis are more active in illuminated material (Introduction and this study), slices of greened leaves were fed in the light. However, many plant tissues rapidly oxidize formate to CO_2 and H_2O. Consequently [^{14}C]formate will produce $^{14}CO_2$ which may be extensively re-fixed in the light. If the re-fixation products are the same as those from direct [^{14}C]formate

FIGURE 6

TIME COURSE OF [^{3}H] FORMATE INCORPORATION BY BARLEY LEAF SEGMENTS

After 15 min incubation period 37.5 μci of sodium [^{3}H]-formate (sp. act. 182 μci/μmole) was fed to 8-day greened leaf segments for up to 30 min in the light (500 μeinsteins m^{-2} sec^{-1}). Leaf extraction, amino acid fraction separation and analysis was as described in the Materials and Methods. Each data point represents a mean value ± S.E.M., obtained from duplicate determinations performed on each of three separate experiments.

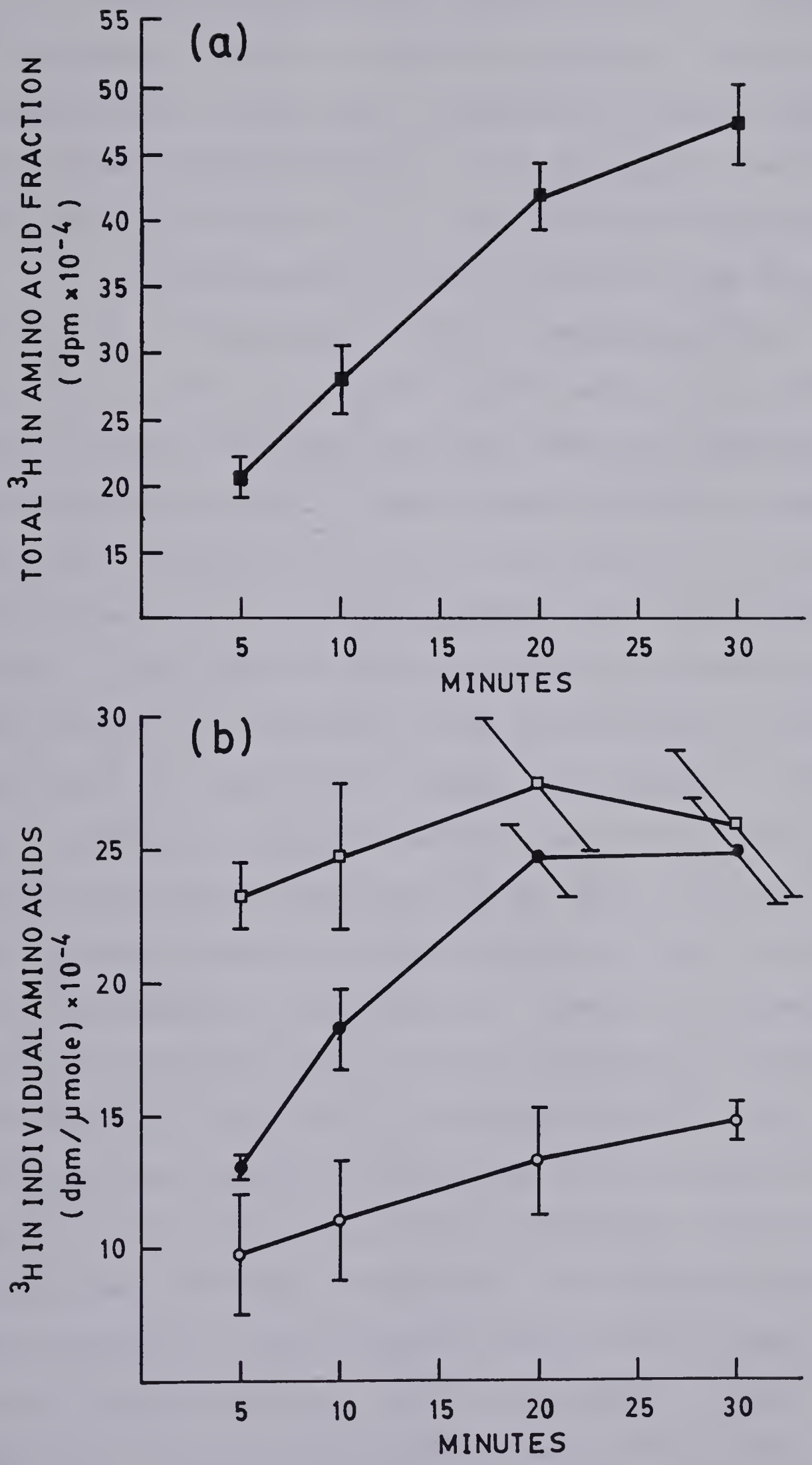
(a) total [^{3}H] incorporated into the free amino acid pool

(b) [^{3}H] incorporated into individual amino acids

—□— serine

—●— glycine

—○— aspartic acid


(a)
TOTAL ^3H IN AMINO ACID FRACTION
(dpm ×10^{-4})
55
50
45
40
35
30
25
20
15
5
10
15
20
25
30
MINUTES
(b)
^3H IN INDIVIDUAL AMINO ACIDS
(dpm/μmole) ×10^{-4}
30
25
20
15
10
5
10
15
20
25
30
MINUTES

metabolism, erroneous information may be obtained regarding the importance of the 10-formyltetrahydrofolate synthetase reaction. To overcome this, [^{3}H]formate was supplied in the greening studies. In such cases formate oxidation would produce ^3H_2O. To determine the possible significance of ^3H_2O exchange (Humphrey and Davies, 1975), as opposed to metabolism as a ^{3}HCO-unit, the products of [^{3}H] - and [^{14}C]formate metabolism were compared.

The distribution of label in various cellular fractions is shown in Table 4. In both cases a significant proportion of the label entered the amino acid fraction. Typical labelled amino acid profiles, obtained from analysis of the neutral and acidic amino acids (Materials and Methods), are presented in Figure 7. These analyses resulted in between 75-90% of the label in the amino acid fraction being recovered in a few clearly resolved amino acids; typically aspartate, serine and glycine for [^{3}H]formate feeding. Minor amounts of label occurred throughout the elution profile, these however were consistently below twice background and not considered significant (see Materials and Methods). The feeding of either radioisotope produced no significant label in either the basic amino acids, or adenine which commonly separates with the latter. The data (Table 4) show that glycine and serine were major components of the labelled amino acid fraction regardless of the isotope fed. However [^{14}C]formate feeding resulted in the additional labelling of glutamate and alanine. This reflected $^{14}CO_2$ re-fixation, as indicated by the $^3H/^{14}C$ ratio. In this experiment equal contributions of ^{3}H and ^{14}C to the labelled products would result in a ratio of 3 as the amount of [^{3}H]formate supplied was three-fold higher than [^{14}C]formate. Significant $^{14}CO_2$ re-fixation would reduce this value especially if the re-fixation products were the same as those from direct formate metabolism. In all of the products isolated from the greened material, it is clear that significant re-fixation of $^{14}CO_2$ occurred (Table

Table 4. Comparison of products formed from [^{3}H]- and [^{14}C] formate fed to eight-day barley leaf sections.

	8 day greened material			8 day etiolated material		
	[^{3}H] formate	[^{14}C] formate	^{3}H/^{14}C	[^{3}H] formate	[^{14}C] formate	^{3}H/^{14}C
Ether solubles	42.1 *± 3.5*	48.0 *± 2.5*	0.9	8.4 *± 0.7*	10.7 *± 2.0*	0.8
Sugars	147.2 *± 4.7*	437.3 *± 14.8*	0.3	44.3 *± 0.4*	16.6 *± 1.6*	2.7
Organic acids	822.3 *± 47.9*	626.8 *± 21.8*	1.3	785.0 *± 31.2*	216.4 *± 18.6*	3.6
Soluble amino acids						
(a) total pool	543.1 *± 90.8*	848.2 *± 18.3*	0.6	165.2 *± 17.0*	267.0 *± 18.1*	0.6
(b) individual components						
aspartic acid	84.0 *± 25.7*	146.5 *± 22.3*	0.6	15.0 *± 6.1*	51.7 *± 2.2*	0.3
serine	220.0 *± 22.6*	297.0 *± 6.6*	0.7	65.8 *± 6.3*	59.7 *± 6.6*	1.1
glutamic acid	*n.d.*	31.3 *± 3.3*		*n.d.*	29.6 *± 3.5*	
glycine	156.8 *± 9.5*	146.1 *± 25.7*	1.1	25.6 *± 4.6*	19.7 *± 0.3*	1.3
alanine	*n.d.*	142.8 *± 3.8*		*n.d.*	12.8 *± 1.4*	
methionine	*n.d.*	*n.d.*		*n.d.*	5.0 *± 1.5*	

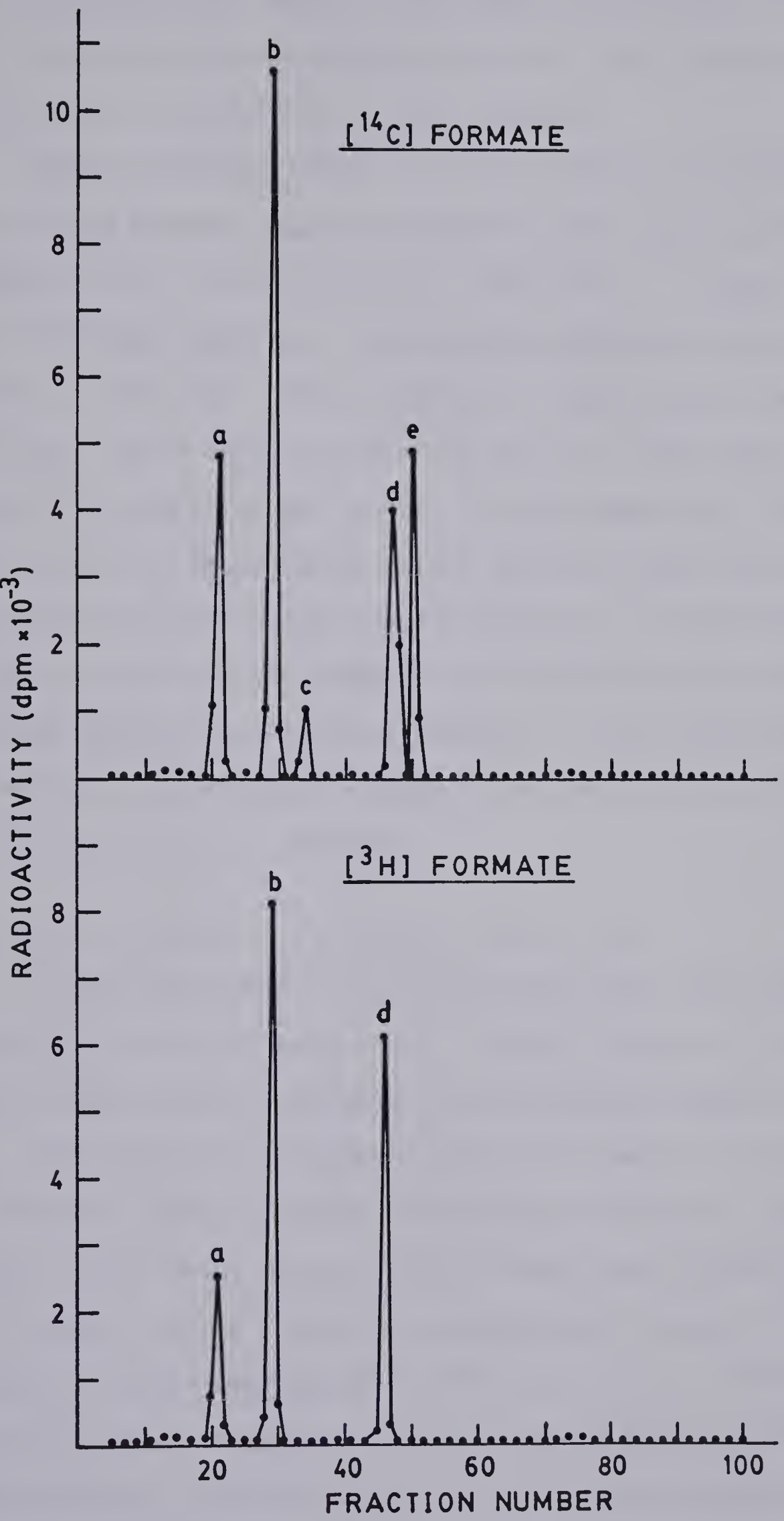
n.d. not detected. 10^{-3} x radioactivity recovered (dpm/3 leaves) ± S.E.M. Leaf tissue and general procedures were as outlined for Figure 6. ^{3}H/^{14}C is the ratio of dpm incorporated from [^{3}H] formate to that from [^{14}C] formate feeding.

FIGURE 7

TYPICAL [^{14}C]- AND [^{3}H]-LABELLED AMINO ACID PROFILES, OBTAINED AFTER AMINO ACID ANALYSIS

After a 15 min pre-incubation period 37.5 µci of sodium [^{3}H] formate (sp. act. 182 µci/µmole) or 12.5 µci of sodium [^{14}C] formate (60.7 µci/mole) were fed to eight-day old barley leaf sections for 20 min. Feeding to greened material was in the light (500 µeinsteins m^{-2} sec^{-1}), and in the dark to etiolated sections. Leaf extraction, ion-exchange fractionation, and amino acid analysis was as described in the Materials and Methods. Each data point represents a mean value ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

a-aspartic acid, b-serine, c-glutamic acid, d-glycine, e-alanine


[14C] FORMATE
[3H] FORMATE
a
b
c
d
e
RADIOACTIVITY (dpm ×10-3)
FRACTION NUMBER
2
4
6
8
10
20
40
60
80
100

4). This was particularly marked in the sugar fraction where the ratio was 0.3. Considerably less re-fixation occurred in the etiolated controls, where feeding was carried out in the dark (Table 4).

Humphrey and Davis (1975) have shown that plant transaminases actively catalyse exchange reactions between ^3H (from ^3H_2O) and the hydrogen attached to the α-carbon atom of most amino acids. In leaf tissue exposed to ^3H_2O they showed that considerable labelling of most soluble amino acids occurred after a 20 min exposure. Significantly, labelling of glycine was some 40 times less than that shown by other amino acids, including serine, glutamate and alanine. In the present work (Table 4), the approximately 1:1 relationship between labelled glycine and serine, and the presence of label in only one other amino acid, asparatate, strongly argues against significant ^3H exchange via transamination reactions. This, and the close theoretical relationship between the amino acids labelled and C-1 metabolism (Introduction), suggests that metabolism of [^3H]formate occurred via activation of a ^3HCO-unit.

The metabolism of [^3H]formate by greening barley leaves

In these experiments, six-day etiolated leaves were either greened or held in the dark as controls. Labelled formate was fed to sections of leaves obtained throughout the study period (Materials and Methods). The distribution of label in the various cellular fractions is shown in Figure 8. Greening leaves incorporated significantly more label into sugars, soluble amino acids and ether solubles than did the controls. In these fractions, maximal levels were reached after 2 days of greening, with greatest increases occurring during the first 24 hrs of illumination. In contrast the amount of label entering the protein amino acid fraction declined rapidly over the same period. Although significant labelling of

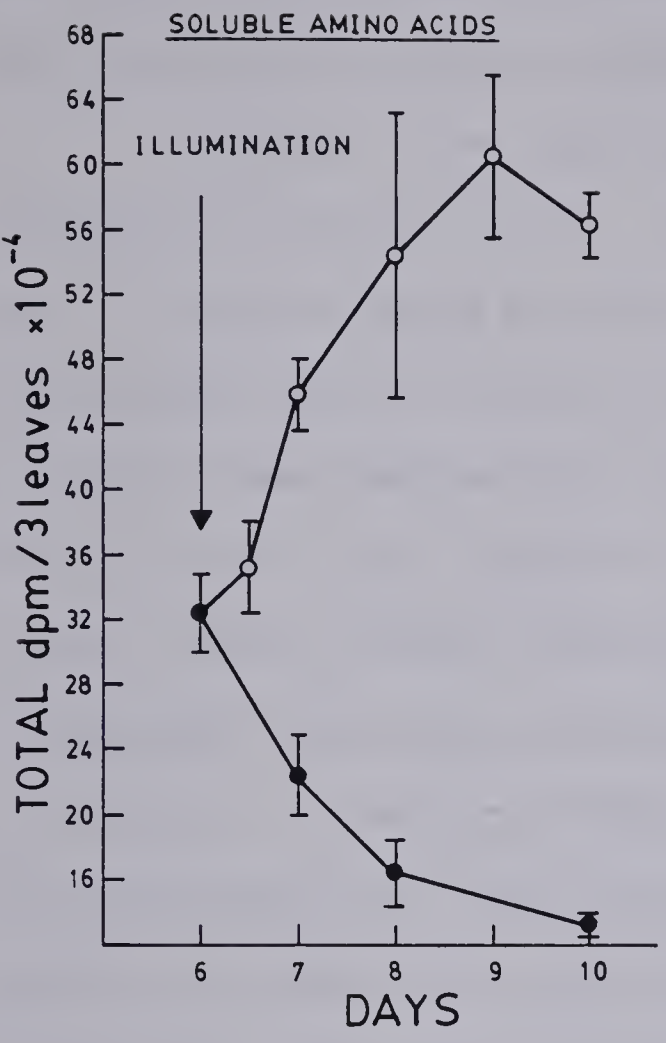
FIGURE 8

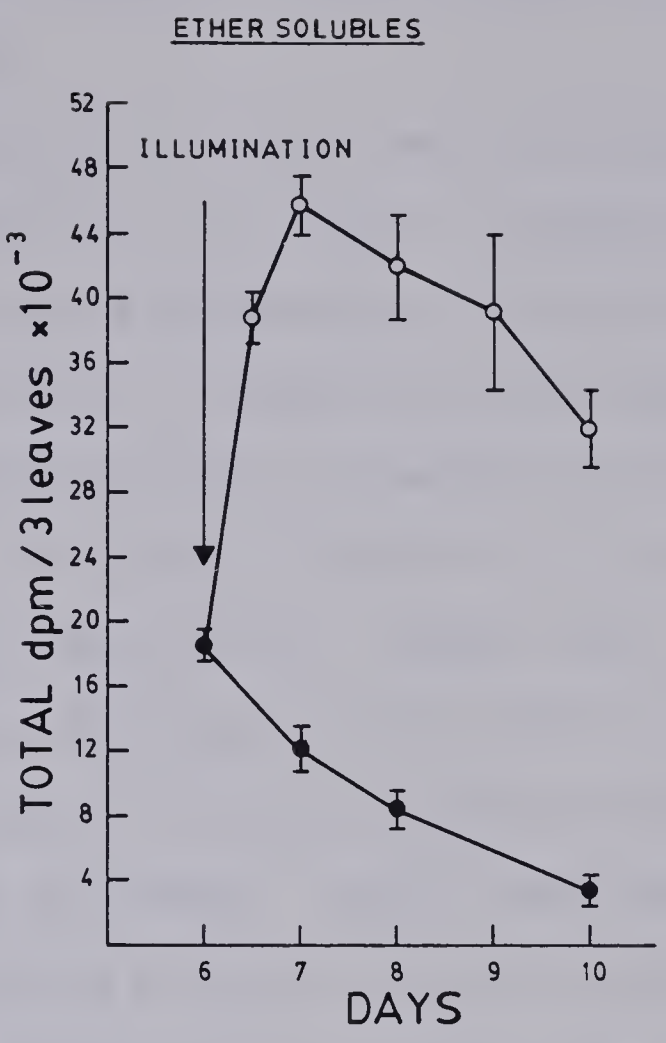
[^{3}H] FORMATE INCORPORATION INTO GENERAL METABOLIC POOLS DURING GREENING

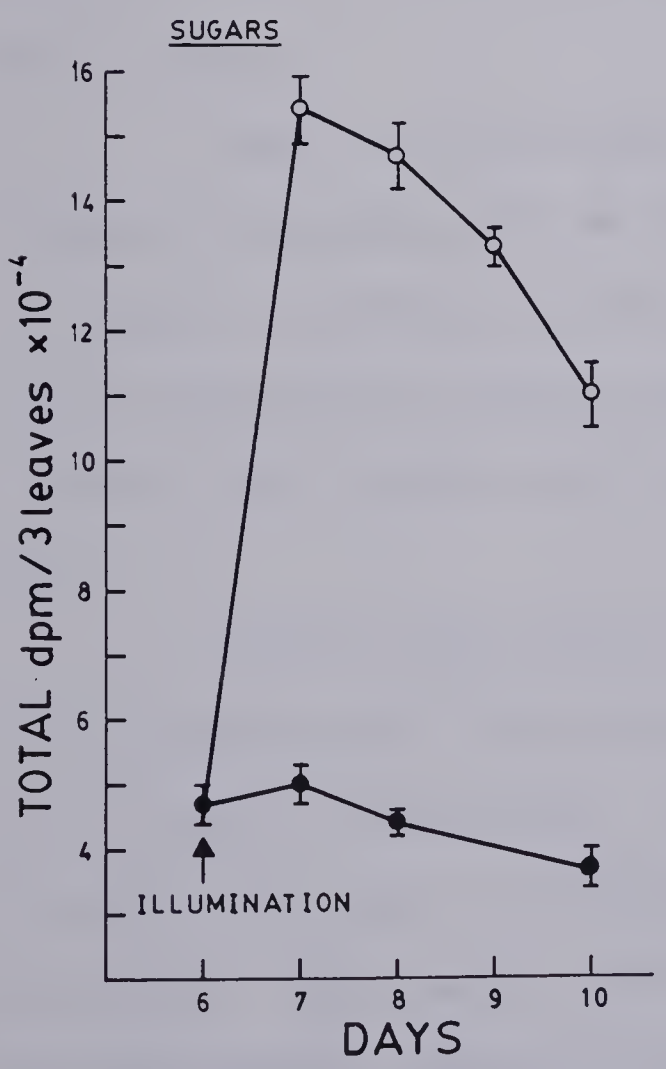
Procedures followed were those outlined in the Materials and Methods. Six-day old barley leaves were either greened or held in the dark as controls. Samples were taken throughout a four day treatment period. After a 15 min pre-incubation 37.5 μci of sodium [^{3}H] formate (sp. act. 182 μci/μmole) were fed to greened leaf segments for 20 min in the light (500 μeinsteins m^{-2} sec^{-1}). Pre-incubation and feeding to etiolated controls was in the dark. Leaf segments were extracted, and the extract fractionated by ion-exchange chromatography. Each data point represents a mean value ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

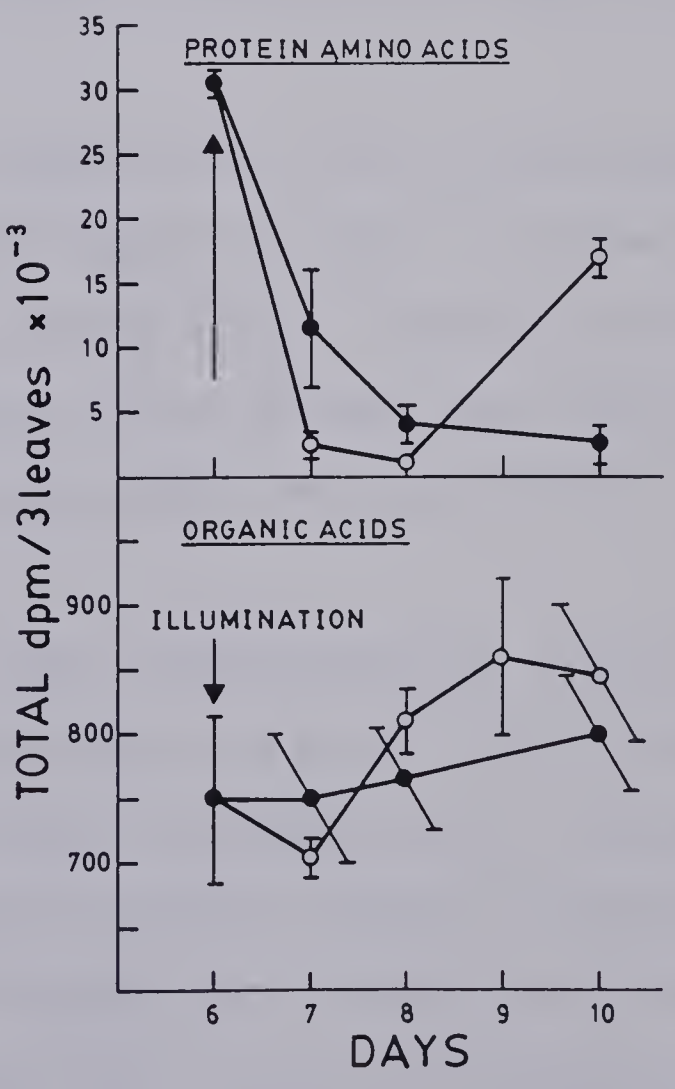
—○— greened material

—●— etiolated controls


SOLUBLE AMINO ACIDS
ILLUMINATION
TOTAL dpm/3leaves ×10⁻⁴
68
64
60
56
52
48
44
40
36
32
28
24
20
16
6
7
8
9
10
DAYS


ETHER SOLUBLES
ILLUMINATION
TOTAL dpm/3leaves ×10⁻³
52
48
44
40
36
32
28
24
20
16
12
8
4
6
7
8
9
10
DAYS


SUGARS
TOTAL dpm/3leaves ×10⁻⁴
16
14
12
10
8
6
4
ILLUMINATION
6
7
8
9
10
DAYS


PROTEIN AMINO ACIDS
ORGANIC ACIDS
ILLUMINATION
TOTAL dpm/3leaves ×10⁻³
35
30
25
20
15
10
5
900
800
700
6
7
8
9
10
DAYS

the organic acid fraction occurred, differences between greened and etiolated material were negligible.

Changes in the labelling of the soluble amino acid fraction are summarized in Figures 8 and 9. These data, expressed on a fresh weight basis, show that during the first 24 hrs of greening, glycine exhibited the greatest rate of labelling increase. In addition glycine became increasingly more important as a labelled product, accounting for 23% to 41% of the total dpm recovered in the amino acid fraction, over the same period. Serine, however declined from 62% to 45%. Maximal levels of incorporation into both glycine and serine occurred after 48 hrs of illumination. Labelling of aspartate was less but increased gradually becoming maximal only after 3 days of greening. Similar trends were observed when these data were expressed as specific radioactivities (Figure 10). Here the rapid increase in specific activity, shown by serine, was due in part to a decrease in pool size (Appendix B), which occurred during greening.

Labelling of individual protein amino acids was also examined (Table 5). Methionine was the major amino acid labelled, but was only detected in etiolated and ten-day greened leaves. Comparable data for seven- and eight-day greened leaves were not obtained due to the very low levels of label incorporated into the protein amino acid fraction (Figure 8).

Overall these data show that formate metabolism is more rapid in the greening leaf, with maximal rates being established during the first 2 days of illumination. It should be emphasized that this period coincides with that for protein and chlorophyll synthesis, and for enzyme activation. Additionally glycine appears to be a major sink for carbon flow from formate, especially during the first 24 hrs of greening.

FIGURE 9

[^{3}H] FORMATE INCORPORATION INTO INDIVIDUAL AMINO ACIDS DURING GREENING

Leaf tissue and general procedures were as outlined in Figure 8. Amino acid fractionation and analysis are as outlined in the Materials and Methods. Each data point represents a mean value ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

—○— greened material

—●— etiolated controls

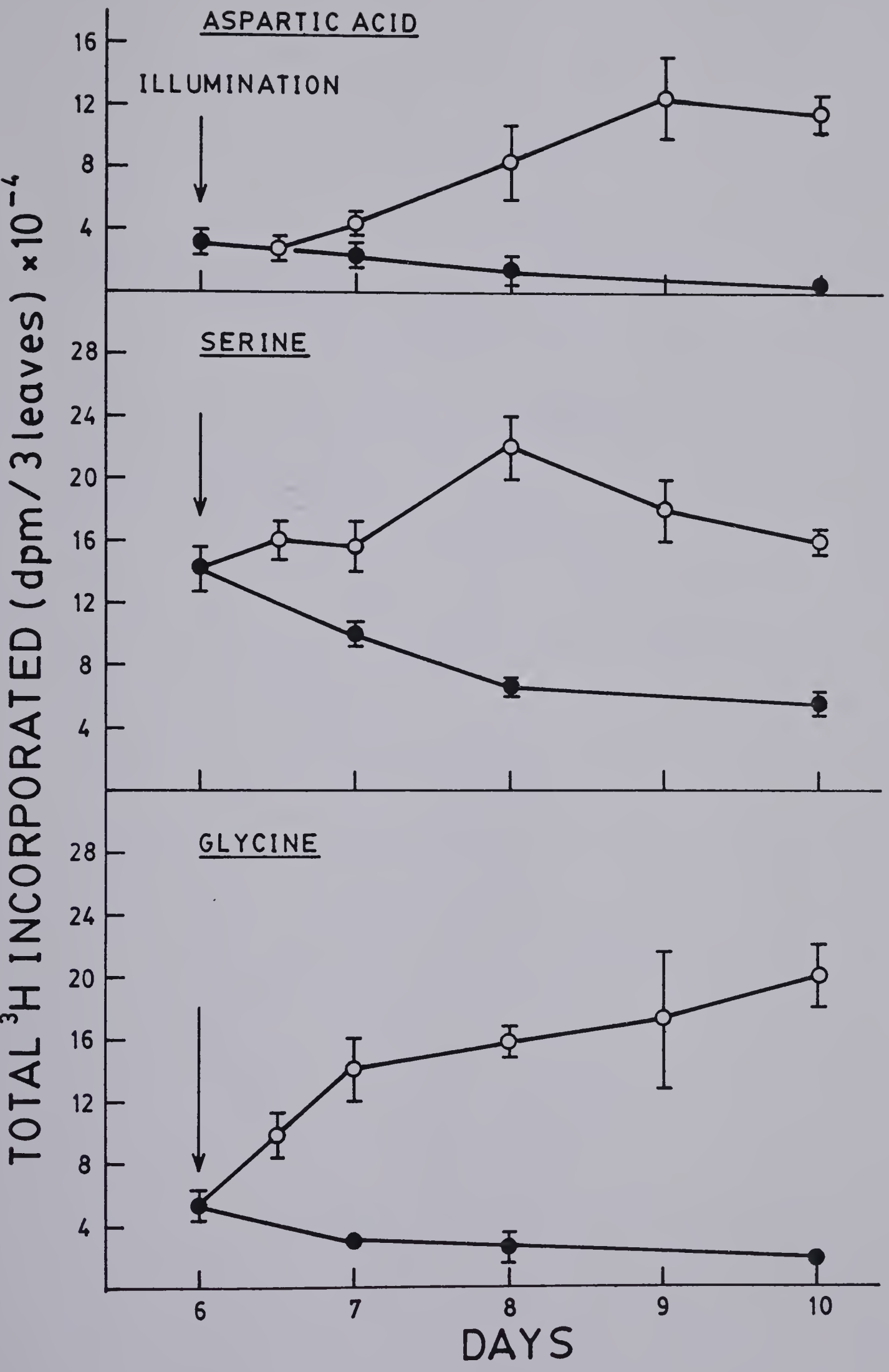

ASPARTIC ACID
ILLUMINATION
SERINE
GLYCINE
TOTAL ^{3}H INCORPORATED (dpm/3 leaves) ×10^{-4}
DAYS
16
12
8
4
28
24
20
16
12
8
4
6
7
8
9
10

FIGURE 10

[^{3}H] FORMATE FEEDING: CHANGES IN SPECIFIC ACTIVITY OF INDIVIDUAL AMINO ACIDS DURING GREENING

For details of tissue and feeding method see Figures 8 and 9. Each data point represents a mean value ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

—○— greened material

—●— etiolated controls

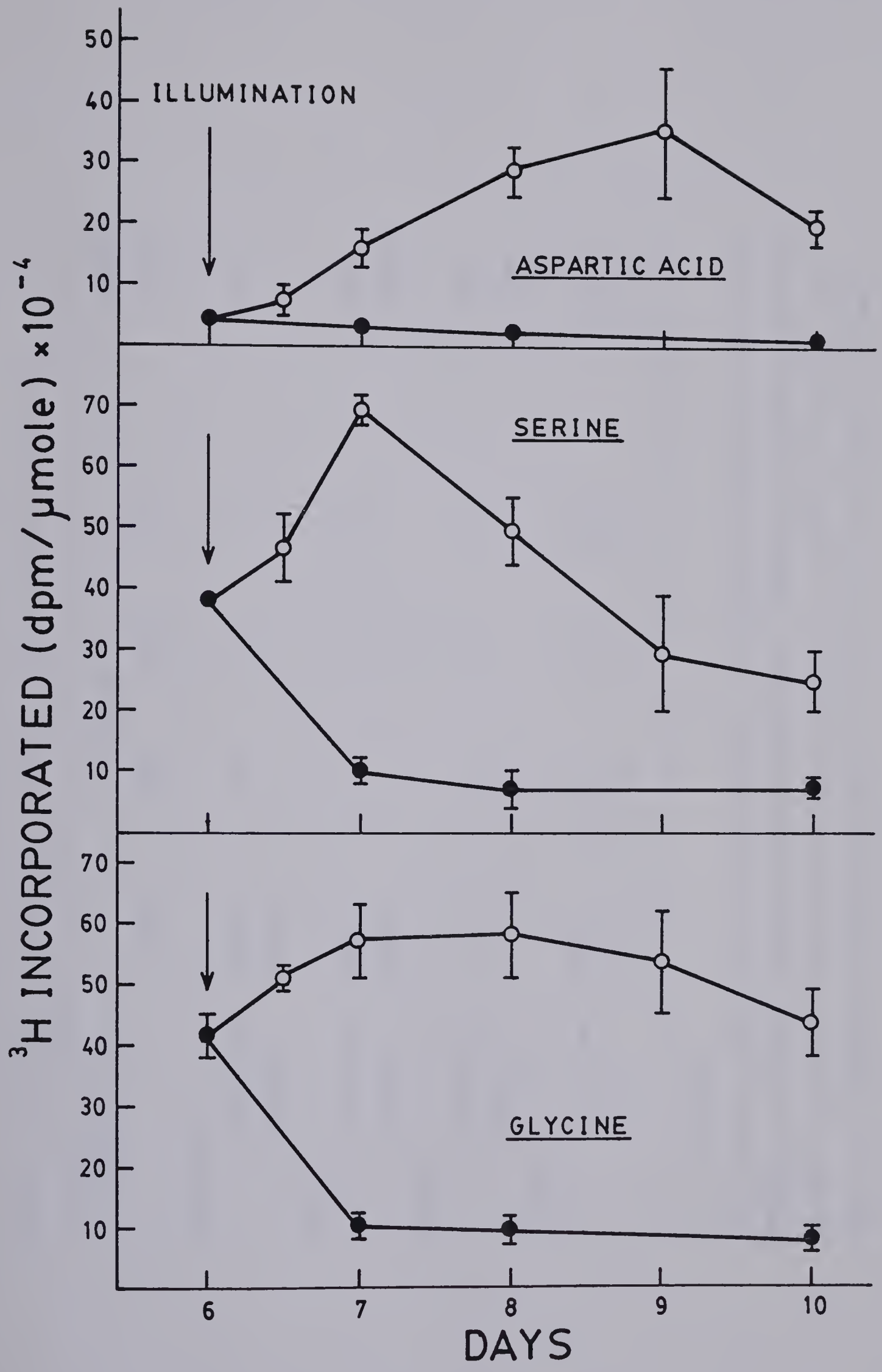

ILLUMINATION
ASPARTIC ACID
SERINE
GLYCINE
^{3}H INCORPORATED (dpm/μmole) ×10^{-4}
DAYS
50
40
30
20
10
70
60
50
40
30
20
10
6
7
8
9
10

Table 5. Incorporation of [^{3}H] formate into individual protein amino acids during greening

Amino acid		Etiolated				Greened
		6 days	7 days	8 days	10 days	10 days
Aspartic Acid						
	dpm/3 leaves	972±26	*n.d.*	*n.d.*	*n.d.*	*n.d.*
	dpm/μmole	429±34				
Serine						
	dpm/3 leaves	3,138±167	*n.d.*	*n.d.*	*n.d.*	1,083±123
	dpm/μmole	2,345±96				405±100
Glycine						
	dpm/3 leaves	*n.d.*	*n.d.*	*n.d.*	*n.d.*	551±37
	dpm/μmole					104±50
Methionine						
	dpm/3 leaves	12,741±447	9,174±673	2,239±426	695±103	12,624±849
	dpm/μmole	61,614±3,778	19,939±2,014	5,670±512	2,518±593	23,762±9,441

n.d. - not detected

Leaf tissue and procedures were as outlined for Figure 8. Acid hydrolysis and amino acid analysis are as per Materials and Methods. Seven-and eight-day greened material incorporated insufficient ^{3}H in the protein amino acid fraction to allow amino acid analysis (see Figure 8). Each data point represents a mean value ± S.E.M., obtained from duplicate determinations performed on each of three separate experiments.

As a corollary to this study [^{3}H]formate metabolism was examined in several other plant species, to determine whether similar trends occurred.

A comparison between [^{3}H]*formate metabolism in barley and other plant species.*

In this study, the isotope was supplied to greened and etiolated leaves. Leaves of barley, corn and dwarfbean, obtained as outlined in the Materials and Methods, were either greened or held in the dark for 1, 2 and 3 days respectively. After these greening periods, chlorophyll contents were approximately 75% of maximum. The broad bean leaves were harvested from mature greenhouse material (Material and Methods). The distribution of label in the various cellular fractions, and in individual soluble amino acids is summarized in Tables 6 and 7. In general terms, these data indicate that formate metabolism was similar in all of the species examined, and was more extensive in the greened material. Specific radioactivities of individual amino acids also reflected this. More specifically however, differences in the degree of labelling of soluble amino acids did occur between the C_3 species and corn. In this connection approximately 40% of the recovered label was in glycine for the greened leaves of the C_3 species examined, but only 6% was recovered in this amino acid for corn. In this C_4 plant, serine was the major labelled amino acid (69%). Also, carbon flow to serine and glycine was similar in corn regardless of treatment. Greened leaves of barley and the other C_3 species on the other hand, showed less serine labelling and three-to four-fold more glycine labelling, when compared to the controls. Clearly at this intermediate stage in greening a major flow of carbon from formate is to serine in corn and to glycine in barley and the other C_3 species examined.

Table 6. [^{3}H] Formate feeding to greened leaf sections from different plant species.

		barley	dwarf bean	broad bean	corn
Ether solubles	dpm	45.8 ± *2.0*	75.8 ± *4.5*	19.4 ± *3.0*	23.4 ± *1.5*
Sugars	dpm	153.8 ± *4.1*	99.0 ± *2.5*	110.9 ± *0.6*	119.7 ± *15.9*
Organic acids	dpm	705.8 ± *18.8*	609.0 ± *54.2*	932.0 ± *2.8*	790.9 ± *80.7*
Soluble amino acids					
(a) total fraction	dpm	411.2 ± *55.2*	777.2 ± *70.7*	470.1 ± *4.0*	646.9 ± *53.4*
(b) individual components					
aspartic acid	sp. act.	159.7 ± *18.9*	103.8 ± *20.3*	49.2 ± *6.8*	94.2 ± *11.3*
	^{3}H (%)	10.8	14.8	5.7	12.2
serine	sp. act.	696.1 ± *29.6*	238.5 ± *33.8*	311.5 ± *16.6*	347.5 ± *86.5*
	^{3}H (%)	38.0	26.4	28.5	69.0
glycine	sp. act.	569.6 ± *92.9*	798.2 ± *71.7*	614.2 ± *35.8*	27.8 ± *4.3*
	^{3}H (%)	33.9	40.5	46.4	6.0
methionine	^{3}H (%)	*n.d.*	5.0	5.7	*n.d.*

n.d. - not detected

dpm - 10^{-3}x radioactivity recovered (dpm/3 leaves) ± S.E.M.

sp. act. - 10^{-3} x specific radioactivity (dpm/µmole) ± S.E.M.

^{3}H (%) - activity as a percent of total ^{3}H in amino acid pool.

Leaves of barley, corn and dwarf bean were greened for 1, 2 and 3 days respectively. Broad bean was greened under conditions outlined in the Materials and Methods. After a 15 min pre-incubation 37.5 µci of [^{3}H] formate (sp. act. 182 µci/µmole) were fed to leaf sect - ions for 20 min. Pre-incubation and feeding was in the light, 500 µeinsteins m^{-2} sec^{-1}. Subsequent extraction, fractionation and amino acid analysis have been described (Materials and Methods). Data represent mean values ± S.E.M., obtained from duplicate determinations on each of two separate experiments.

Table 7. [^{3}H] Formate feeding to etiolated leaf sections from different plant species.

		barley	dwarf bean	corn
Ether solubles	dpm	12.2 ± *1.0*	9.1 ± *1.3*	12.0 ± *0.7*
Sugars	dpm	50.0 ± *0.1*	48.8 ± *2.0*	40.6 ± *2.0*
Organic acids	dpm	746.0 ± *51.0*	105.8 ± *46.3*	470.6 ± *53.2*
Soluble amino acids				
(a) total fraction	dpm	223.1 ± *22.4*	334.1 ± *18.4*	198.3 ± *36.8*
(b) individual components				
aspartic acid	sp. act.	31.7 ± *6.5*	38.9 ± *10.6*	29.9 ± *4.9*
	^{3}H (%)	10.8	19.5	20.6
serine	sp. act.	101.6 ± *16.7*	396.1 ± *81.2*	73.8 ± *25.3*
	^{3}H (%)	45.1	50.9	52.6
glycine	sp. act.	97.1 ± *12.3*	190.7 ± *33.2*	22.8 ± *10.3*
	^{3}H (%)	12.5	14.9	10.2
methionine	^{3}H (%)	*n.d.*	2.7	*n.d.*

Data presentation and general procedures are as outlined for Table 6, except that treatment periods of 1,2 and 3 days respectively for barley, corn and dwarf bean were in the dark, as were the pre-incubation and feeding periods.

Evidence for the activation of formate as a C-1 unit in greening barley

The above feeding experiments indicate that barley leaves can metabolise formate directly as a C-1 unit. In addition, the products of this metabolism, coupled with the presence of active relevant enzymes, suggest that the initial step in this process involves formation of 10-formyltetrahydrofolate. This possibility was examined in further experiments using the folate analogue, aminopterin.

Aminopterin is known to inhibit dihydrofolate reductase activity *in vitro*, and is generally thought to act similarly *in vivo* (Blakley, 1969). As this enzyme reduces H_2PteGlu to H_4PteGlu, tetrahydrofolate deficiency will occur in tissues treated with the analogue (Blakley, 1969; Spronk and Cossins, 1972). In such cases, metabolic reactions dependent upon H_4PteGlu will be severely inhibited. This cascade effect will be most apparent under conditions of rapid folate turnover, that is when the demands for reduced folate derivatives are maximal. Such a period will occur when etiolated leaves green, since green tissues contain considerably higher levels of reduced folates than do their etiolated counterparts (Spronk and Cossins, 1972).

Since the enzymes involved in activating formate and in metabolizing the active C-1 unit require H_4PteGlu (Introduction), their *in vivo* activities will be lower in leaves greened in the presence of aminopterin. Thus if formate is indeed metabolised via this route, significant inhibition should result in the aminopterin treated leaves. To test this, labelled formate was fed to leaf sections which had been greened for 24 hrs in the presence of this analogue (Materials and Methods). Aminopterin was omitted from the controls. In a preliminary experiment (Table 8), inhibition of ^{14}C incorporation occurred in all fractions examined and was dependent upon aminopterin concentration, clearly demonstrating a close association between

Table 8. [^{14}C] Formate metabolism in barley leaves greened in the presence of aminopterin.

	Aminopterin concentration (M)			
	0	10^{-5}	10^{-4}	10^{-3}
Chlorophyll				
mg/g fr. wt.	0.26 ± *0.01*	0.23 ± *0.01*	0.19 ± *0.01*	0.16 ± *0.01*
% inhibition		11.5	26.9	38.5
Ether solubles				
dpm	22.1 ± *0.6*	21.4 ± *0.1*	16.5 ± *0.3*	11.9 ± *0.3*
% inhibition		2.8	25.0	46.3
Sugars				
dpm	260.2 ± *23.0*	231.7 ± *8.2*	145.8 ± *30.4*	128.2 ± *18.5*
% inhibition		11.0	44.0	50.7
Organic acids				
dpm	312.3 ± *14.8*	283.6 ± *10.6*	229.6 ± *27.9*	138.1 ± *18.3*
% inhibition		9.2	26.5	55.8
Soluble amino acids				
dpm	443.1 ± *9.0*	393.3 ± *18.1*	283.3 ± *25.7*	227.1 ± *26.9*
% inhibition		11.2	36.1	48.7
Total ^{14}C incorporated				
dpm	1037.6 ± *47.3*	930.1 ± *37.0*	675.2 ± *84.4*	505.2 ± *64.0*

dpm - 10^{-4} x total ^{14}C incorporated per three leaves ± S.E.M.

Six-day etiolated leaves were greened for 24 hours in the presence of aminopterin (Materials and Methods). After this period 12.5 µci of [^{14}C] formate (sp. act. 60.7 µci/µmole) were fed for 20 min in the light, 500 µeinsteins $m^{-2}sec^{-1}$. This procedure, and those relating to the chlorophyll assay are described in the Materials and Methods. Data represent mean values ± S.E.M., obtained from duplicate determinations on each of two separate experiments.

formate and folate metabolism in greening barley leaves. It should be noted that inherent in this experiment was the assumption that the contribution, via $^{14}CO_2$ re-fixation, to the labelling was not affected by the aminopterin treatment. That this was the case is clear from the data in Table 9, where similar inhibition occurred when [^{3}H]formate was used. In this more detailed analysis, leaves greened in the presence of aminopterin showed a decrease in the specific radioactivity of all soluble amino acid products (Table 9). This suggests that formate, through its activation as a C-1 unit, makes a significant contribution to these cellular pools during greening.

It should also be noted that aminopterin treatment reduce total chlorophyll levels by up to 40% (Table 8).

The effect of INH on formate metabolism in vivo in barley leaves

The formate feeding experiments on barley and other C_3 species (Figures 9 and 10; Table 6), showed glycine to be a major product during greening. If formate is activated as a C-1 unit (Tables 8 and 9), then its subsequent metabolism to glycine will involve a pathway whose final step is catalyzed by glycine synthase (Introduction). This enzyme requires pyridoxal-5^1-phosphate. INH is known to act as a general antagonist of vitamin B_6-requiring enzymes (Hicks and Cymerman-Craig, 1957). Consequently, if formate is metabolized via this route INH treated leaves should have less ability to produce labelled glycine. To test this, [^{3}H]formate was fed to sections of eight-day greened barley leaves which had been preincubated with INH for 60 min (Materials and Methods). The data (Table 10) clearly show that INH reduced glycine labelling by as much as 90%. INH also reduced carbon flow to serine by up to 80%. This is not surprising since synthesis, in the glycollate pathway, involves serine hydroxymethyltransferase, a vitamin B_6-dependent enzyme (Introduction), also sensitive

Table 9. [^{3}H] Formate metabolism in barley leaves greened in the presence of aminopterin.

		Aminopterin concentration (M)	
		0	10^{-4}
Ether solubles	dpm	51.3 ± *0.5*	34.6 ± *1.5*
Sugars	dpm	397.2 ± *47.6*	204.3 ± *40.8*
Organic acids	dpm	648.0 ± *133.1*	468.9 ± *27.9*
Soluble amino acids	dpm	759.6 ± *7.0*	518.0 ± *9.1*
Total [^{3}H] incorporated	dpm	1856.0 ± *188.2*	1225.8 ± *79.3*
total % inhibition			34.0
*Individual amino acids labelled			
aspartic acid	dpm	70.6 ± *16.2*	32.5 ± *4.3*
	sp. act.	144.5 ± *3.4*	23.9 ± *3.1*
	µmoles	0.49 ± *0.10*	1.36 ± *0.13*
serine	dpm	370.6 ± *16.2*	225.4 ± *2.0*
	sp. act.	339.4 ± *14.9*	251.6 ± *2.3*
	µmoles	1.09 ± *0.31*	0.90 ± *0.09*
glutamic acid	dpm	33.3 ± *2.5*	13.5 ± *1.1*
	sp. act.	33.3 ± *2.5*	9.9 ± *0.8*
	µmoles	1.00 ± *0.13*	1.36 ± *0.07*
glycine	dpm	74.4 ± *3.9*	148.7 ± *11.4*
	sp. act.	171.0 ± *4.6*	118.0 ± *7.0*
	µmoles	0.44 ± *0.07*	1.26 ± *0.20*
alanine	dpm	17.0 ± *1.8*	6.3 ± *0.2*
	sp. act.	56.0 ± *6.0*	13.9 ± *0.5*
	µmoles	0.30 ± *0.10*	0.46 ± *0.11*

dpm - 10^{-3}x total ^{3}H incorporated per three leaves ± S.E.M.

sp. act. - 10^{-3} x specific radioactivity (dpm/µmole) ± S.E.M.

µmoles - quantitative pool size per three leaves ± S.E.M.

General procedures were as outlined for Table 8. [^{3}H]Formate (37.5 µci, sp. act. 182 µci/µmole) was fed for 20 min in the light, 500 µeinsteins m^{-2} sec^{-1}. Data represent mean values ± S.E.M., obtained from duplicate determinations on each of two separate experiments.

* The pool sizes of other amino acids were not effected by the treatment

Table 10. Effect of INH on [^{3}H] formate metabolism in greened barley leaves.

		INH concentration (M)		
		0	7.3×10^{-2}	36.0×10^{-2}
Ether solubles	dpm	43.2 ± *3.3*	31.4 ± *1.1*	23.2 ± *2.9*
Sugars	dpm	142.5 ± *5.3*	127.0 ± *15.6*	80.6 ± *4.2*
Organic acids	dpm	822.0 ± *49.8*	771.8 ± *161.9*	973.5 ± *34.3*
soluble amino acids	dpm	522.3 ± *49.2*	352.9 ± *13.5*	232.7 ± *3.5*
Total [^{3}H] incorporated	dpm	1530.0 ± *107.5*	1283.2 ± *192.1*	1310.0 ± *44.9*
total % inhibition			16.1	14.4
Individual amino acids labelled				
aspartic acid	dpm	84.0 ± *25.7*	35.3 ± *7.3*	66.9 ± *2.6*
	sp. act.	290.8 ± *34.5*	178.2 ± *45.9*	290.9 ± *70.8*
	µmoles	0.28 ± *0.06*	0.20 ± *0.01*	0.23 ± *0.06*
serine	dpm	220.0 ± *22.6*	115.5 ± *12.3*	45.9 ± *7.8*
	sp. act.	493.2 ± *51.7*	489.3 ± *5.7*	209.6 ± *21.0*
	µmoles	0.45 ± *0.03*	0.24 ± *0.02*	0.22 ± *0.05*
glycine	dpm	156.8 ± *9.5*	87.5 ± *7.2*	12.8 ± *0.2*
	sp. act.	570.2 ± *75.4*	25.3 ± *2.0*	3.8 ± *0.5*
	µmoles	0.28 ± *0.02*	3.46 ± *0.01*	3.38 ± *0.39*

dpm - 10^{-3}x total ^{3}H incorporated per three leaves ± S.E.M.
sp. act.- 10^{-3}x specific radioactivity (dpm/µmole) ± S.E.M.
µmoles - quantitative pool size per three leaves ± S.E.M.
Leaf sections from 8-day greened material were preincubated with INH for 60 min. [^{3}H] formate (37.5 µci, sp. act. 182 µci/µmole) was fed for 20 min. Preincubation and feeding was in the light, 500 µeinsteins $m^{-2}sec^{-1}$. These procedures, and those relating to the subsequent work-up have beendescribed in the Materials and Methods. Data represent mean values ± S.E.M., obtained from duplicate determinations on each of two separate experiments.

to INH (Miflin *et al.*, 1966). Additionally, the INH treatments resulted in a two-fold decrease in the serine pool, and a greater than ten-fold increase in the glycine pool.

The nature of the glycollate pathway in greening barley leaves

If glycine is an important metabolic product during greening, then carbon flow in the glycollate pathway might not involve the more customary glycine→serine→sugars interconversions (Introduction). To examine this possibility various pathway intermediates were fed in the light to sections of seven-day greened leaves (Materials and Methods). This stage of greening was chosen because carbon flow to glycine from formate was maximal.

To assess overall carbon flow in the glycollate pathway [2-^{14}C] glycollate (Ca salt) was fed. The data (Table 11) show that more than 75% of the metabolised glycollate was recovered in the soluble amino acid fraction, of which 90% was in glycine and only 4% in serine. These data, coupled with the slow labelling of the sugar fraction (13% recovered) show that glycine is a sink for carbon flowing from glycollate and imply that flow from glycine to serine and sugars is restricted in greening barley leaves. This latter conclusion raised questions concerning the activities of serine hydroxymethyltransferase and glycine synthase during this period (Introduction).

Glycine cleavage was examined by feeding equimolar amounts of [1-^{14}C]- and [2-^{14}C]glycine (Materials and Methods). In either case less than 5% of the glycine was metabolised (Table 12). Some serine labelling was detected, and the 2:1 ratio of dpm incorporated from [2-^{14}C]glycine compared to [1-^{14}C]glycine indicated the presence of coupled glycine synthase and serine hydroxymethyltransferase activity. However this activity

Table 11. [2-^{14}C]Glycollate metabolism in partially greened barley leaves.

	Cellular fractions			
	Ether solubles	Sugars	Protein amino acids	Soluble amino acids
dpm	121.4 ± *20.7*	162.3 ± *19.5*	7.2 ± *0.6*	938.5 ± *107.3*

	Individual soluble amino acids		
	aspartic acid	serine	glycine
dpm	25.5 ± *5.4*	37.4 ± *3.1*	870.0 ± *99.5*
sp. act.	109.6 ± *24.8*	147.3 ± *29.5*	4218.2 ± *712.5*

dpm - 10^{-3} x total ^{14}C incorporated per three leaves ± S.E.M.

sp. act.- 10^{-3} x specific radioactivity (dpm/μmole) ± S.E.M.

After 15 min preincubation 2 μci of [2-^{14}C]glycollate (sp. act. 55 μci/μmole) were fed to sections of 7-day greened leaves for 20 min. Preincubation and feeding was in the light, 500 μeinsteins m^{-2} sec^{-1}. These procedures, and those relating to the subsequent work-up have been described (Materials and Methods). Data represent mean values ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

Table 12. [1-^{14}C], and [2-^{14}C]Glycine metabolism in partially greened barley leaves.

		[1-^{14}C]glycine	[2-^{14}C]glycine
Ether solubles	dpm	97.1 ± *5.1*	97.2 ± *11.8*
Sugars	dpm	320.6 ± *15.8*	251.6 ± *5.8*
Organic acids		638.0 ± *10.6*	558.1 ± *7.3*
Soluble amino acids labelled			
unknown (fract. #17)	dpm	32.8 ± *19.3*	67.7 ± *33.9*
aspartic acid	dpm	69.0 ± *13.1*	57.6 ± *3.5*
	sp. act.	160.9 ± *26.0*	59.0 ± *10.1*
serine	dpm	296.2 ± *81.3*	570.4 ± *89.3*
	sp. act.	351.9 ± *61.4*	695.5 ± *30.8*

dpm - 10^{-2} x total ^{14}C incorporated per three leaves ± S.E.M.

sp. act. - 10^{-2} x specific radioactivity (dpm/μmole) ± S.E.M.

After a 15 min preincubation either 1.04 μci of [1-^{14}C]glycine (sp. act. 54 μci/μmole) or 1.0 μci of [2-^{14}C]glycine (sp. act. 52 μci/μmole) were fed to sections of seven-day greened leaves for 20 min. Preincubation and feeding was in the light, 500 μeinsteins m^{-2} sec^{-1}. These procedures, and those relating to the subsequent work-up have been described (Materials and Methods). Data represent mean values ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

was low, when one considers the large amounts of glycine remaining after the feeding period. In keeping with this, little serine hydroxymethyl-transferase activity was detected in leaf extracts (Table 13).

To assess carbon flow from serine other feeding experiments were carried out with [3-^{14}C]serine (Materials and Methods). Surprisingly less than 3% of the amino acid was metabolised, and little labelling of the sugar fraction was observed (Table 14). In contrast Wang and Burris (1963) showed that both [3-^{14}C]- and [1-^{14}C]serine were rapidly metabolized to sugars and organic acids by mature green wheat leaves, when fed under similar conditions.

An examination of glycollate as a precursor of formate in greening barley leaves

Enzyme studies of greening barley leaves (Figures 2 and 3), suggested a possible *in vivo* carbon flow from glycollate to formate, via glyoxylate decarboxylation. In this regard it is envisaged that formate carbon arises from the C_2 of glycollate, the C_1 being lost as CO_2. An attempt was therefore made to evaluate the flow of carbon through this pathway by preincubating sections of seven-day greened leaves with carrier formate, prior to [2-^{14}C]glycollate feeding. Under these conditions [^{14}C]-formate, generated from [2-^{14}C]glycollate, will be diluted by the unlabelled carrier resulting in decreased labelling if significant carbon flow to formate is occurring. The results are shown in Table 15.

While overall incorporation of ^{14}C was not effected by the treatments, the labelling of the ether soluble fraction decreased by up to 75%. A detailed analysis of the amino acid fraction revealed decreases in specific radioactivities of all amino acids labelled. However these were not caused so much by a decrease in labelled carbon flow but rather by an increase in the overall pool size, in response to treatment.

Table 13. Serine hydroxymethyltranferase activity in partially greened barley leaves.

	nmoles product/min/g fresh wt.	nmoles product/min/mg protein
Crude extract	0.84 ± *0.08*	0.04 ± *0.01*
De-salted extract	1.07 ± *0.11*	0.07 ± *0.01*

Six-day etiolated leaves were greened for 24 hours at 500 μeinsteins m^{-2} sec^{-1}, and extracted. The complete reaction system (final pH 8.0) and assay procedures are described in the Materials and Methods. Data represent mean values ± S.E.M. obtained from duplicate assays on each of two separate experiments.

Table 14. [3-^{14}C]Serine metabolism in partially greened barley leaves.

		1µci	5µci
Ether solubles	dpm	87.8 ± 10.6	1705.3 ± 80.3
Sugars	dpm	279.3 ± 47.0	1218.3 ± 327.6
Soluble amino acids labelled			
aspartic acid	dpm	107.9 ± 17.5	445.2 ± 59.1
	sp. act.	531.5 ± 15.9	1601.4 ± 382.7
	µmoles	0.20 ± 0.03	0.28 ± 0.04
glycine	dpm	67.5 ± 8.1	348.9 ± 59.1
	sp. act.	262.5 ± 11.4	586.4 ± 36.7
	µmoles	0.26 ± 0.04	0.60 ± 0.06
alanine	dpm	170.2 ± 19.5	843.9 ± 96.6
	sp. act.	143.7 ± 5.7	440.0 ± 11.0
	µmoles	1.19 ± 0.09	1.92 ± 0.17

dpm - 10^{-2} x total ^{14}C incorporated per three leaves ± S.E.M.

sp. act. - 10^{-2} x specific radioactivity (dpm/µmole) ± S.E.M.

µmoles- quantitative pool size per three leaves ± S.E.M.

After 15 min preincubation either 1 µci or 5 µci of [3-^{14}C]serine (sp. act. 54 µci/µmole) were fed to sections of seven-day greened leaves for 20 min. Preincubation and feeding was in the light, 500 µeinsteins m^{-2} sec^{-1}. These procedures, and those relating to the subsequent work-up have been described (Materials and Methods). Data represent mean values ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

Table 15. Effect of formate on [2-^{14}C]glycollate metabolism in partially greened barley leaves.

		Sodium formate concentration (M)		
		0	6.0×10^{-4}	6.0×10^{-3}
Ether solubles	dpm	121.4 ± *20.7*	52.7 ± *18.6*	28.5 ± *6.0*
Sugars	dpm	162.3 ± *19.5*	153.5 ± *26.5*	176.1 ± *73.8*
Protein amino acids	dpm	7.2 ± *0.6*	5.6 ± *0.5*	5.2 ± *0.1*
Soluble amino acids	dpm	938.5 ± *107.3*	1178.3 ± *62.4*	976.9 ± *158.1*
Total [^{14}C] incorporated	dpm	1229.4 ± *148.1*	1390.1 ± *108.0*	1186.7 ± *238.0*
*Individual amino acids labelled.				
aspartic acid	dpm	25.5 ± *5.4*	14.5 ± *4.1*	9.7 ± *2.1*
	sp. act.	109.6 ± *24.8*	46.8 ± *4.0*	28.6 ± *4.6*
	µmoles	0.25 ± *0.05*	0.31 ± *0.06*	0.36 ± *0.10*
serine	dpm	37.4 ± *3.1*	50.9 ± *7.9*	50.3 ± *11.0*
	sp. act.	147.3 ± *29.5*	113.7 ± *18.1*	75.3 ± *14.9*
	µmoles	0.27 ± *0.03*	0.47 ± *0.04*	0.73 ± *0.19*
glycine	dpm	870.0 ± *99.5*	1135.7 ± *98.1*	838.6 ± *159.7*
	sp. act.	4218.2 ± *112.5*	2880. ± *118.3*	2473.8 ± *210.1*
	µmoles	0.22 ± *0.03*	0.40 ± *0.11*	0.34 ± *0.05*

dpm - 10^{-3} x total ^{14}C incorporated per three leaves ± S.E.M.

sp. act. - 10^{-3} x specific radioactivity (dpm/µmoles) ± S.E.M.

µmoles - quantitative pool size per three leaves ± S.E.M.

Leaf sections from seven-day greened material were preincubated with sodium formate for 20 min. [2-^{14}C]Glycollate (2 µci, sp. act. 55 µci/µmole) was fed for 20 min. Preincubation and feeding was in the light, 500 µeinsteins m^{-2} sec^{-1}. These procedures and those relating to the subsequent work-up have been described (Materials and Methods). Data represent mean values ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

* The pool sizes of other amino acids were not effected by the treatment.

Formate production from glycollate, via glyoxylate, is closely coupled to the activity of glycollate oxidase (Introduction). This enzyme is competitively inhibited by αHPMS (Zelitch, 1957; Corbett and Wright, 1971), a compound which effectively reduces carbon flow in the glycollate pathway (Zelitch, 1975). The effect of this inhibitor on formate metabolism was examined. In these experiments seven-day etiolated leaves were greened for 24 hrs in the presence of αHPMS, prior to formate feeding. In a preliminary study (Table 16) inhibition of [^{14}C]formate incorporation occurred in all fractions examined, and was dependent upon αHPMS concentration. In addition chlorophyll synthesis was greatly reduced in the treated leaves. A more detailed analysis, using [^{3}H]formate (Table 17), showed that significant inhibition in the labelling of glycine and serine occurred in response to treatment. These data imply a flow of carbon *in vivo* from glycollate to formate in greening barley leaves. Further they suggest that the formate activating enzyme, 10-formyltetrahydrofolate synthetase, may be regulated by formate, either through activation or *de novo* synthesis. Consistent with this suggestion, analysis of synthetase levels (Table 18) showed less activity when crude or de-salted extracts of α-HPMS-treated leaves were examined.

Table 16. [^{14}C]Formate metabolism in barley leaves greened in the presence of αHPMS.

	αHPMS concentration (M)			
	0	10^{-4}	10^{-3}	10^{-2}
Chlorophyll				
mg/g fr. wt.	0.64 ± *0.01*	0.54 ± *0.02*	0.40 ± *0.01*	0.13 ± *0.01*
% inhibition		15.6	37.5	79.7
Ether solubles				
dpm	29.4 ± *3.5*	23.5 ± *1.0*	17.1 ± *5.3*	4.8 ± *0.5*
% inhibition		20.0	41.8	83.7
Sugars				
dpm	178.0 ± *14.2*	151.1 ± *10.2*	122.2 ± *10.5*	44.5 ± *7.4*
% inhibition		15.1	31.4	75.0
Organic acids				
dpm	314.3 ± *4.8*	277.5 ± *9.9*	250.9 ± *24.7*	156.6 ± *16.6*
% inhibition		11.7	20.7	50.2
Soluble amino acids				
dpm	343.5 ± *9.9*	272.1 ± *11.0*	234.4 ± *8.7*	103.9 ± *16.5*
% inhibition		20.8	31.8	69.7
Total ^{14}C incorporated				
dpm	865.2 ± *32.4*	724.2 ± *32.1*	624.4 ± *49.2*	309.8 ± *41.0*

dpm - 10^{-3} x total ^{14}C incorporated per three leaves ± S.E.M.

Seven-day old etiolated leaves were greened for 24 hours in the presence of αHPMS (Materials and Methods). After a 15 min preincubation 12.5 μci of [^{14}C]formate (sp. act. 60.7 μci/μmole) were fed to sections of the greened leaves for 20 min. Preincubation and feeding was in the light, 500 μeinsteins m^{-2} sec^{-1}. These procedures, and those relating to the chlorophyll assay, are described in the Materials and Methods. Data represent mean values ± S.E.M., obtained from duplicate determinations on each of two separate experiments.

Table 17. [^{3}H]Formate metabolism in barley leaves greened in the presence of αHPMS

		αHPMS concentration (M)	
		0	10^{-3}
Ether solubles	dpm	263.1 ± *13.6*	208.3 ± *9.2*
Sugars	dpm	90.8 ± *10.1*	36.0 ± *0.1*
Organic acids	dpm	536.8 ± *29.4*	423.0 ± *10.1*
Soluble amino acids	dpm	503.6 ± *8.6*	356.7 ± *4.1*
Total [^{3}H] incorporated	dpm	1394.3 ± *61.7*	1024.0 ± *23.5*
	total % inhibition		26.6
Individual amino acids labelled			
aspartic acid	dpm	98.1 ± *3.1*	95.6 ± *3.2*
	sp. act.	171.6 ± *5.5*	190.1 ± *6.3*
serine	dpm	225.8 ± *5.8*	143.4 ± *2.4*
	sp. act.	294.8 ± *7.5*	191.5 ± *3.2*
glycine	dpm	54.3 ± *2.1*	35.8 ± *1.1*
	sp. act.	189.2 ± *7.2*	142.5 ± *4.4*
alanine	dpm	8.0 ± *1.2*	7.8 ± *0.3*
	sp. act.	13.1 ± *1.9*	10.8 ± *0.4*

dpm - 10^{-3} x total ^{3}H incorporated per three leaves ± S.E.M.

sp. act. - 10^{-3} x specific radioactivity (dpm/μmole) ± S.E.M.

General procedures were as outline for Table 16. Illuminated preincubation and feeding periods were 15 and 20 min respectively. The amount of [^{3}H]formate fed was 37.5 μci (sp. act. 182 μci/μmole). Data represent mean values ± S.E.M., obtained from duplicate determinations on each of two separate experiments.

Table 18. 10-Formyltetrahydrofolate synthetase activity in barley leaves, greened in the presence of αHPMS.

	αHPMS concentration (M)		
	0	10^{-3}	10^{-2}
Crude extract			
nmoles/min/g fresh wt.	101.1 ± 6.3	97.1 ± 2.0	70.2 ± 2.3
% inhibition		4.0	30.6
nmoles/min/mg protein	6.9 ± 0.5	6.2 ± 0.2	4.3 ± 0.1
% inhibition		10.1	37.7
De-salted extract			
nmoles/min/g fresh wt.	228.2 ± 6.5	226.6 ± 8.2	172.9 ± 6.6
% inhibition		0.7	24.2
nmoles/min/mg protein	28.9 ± 0.7	27.2 ± 0.7	22.1 ± 0.6
% inhibition		5.9	23.5

General procedures were as outlined for Table 16. The complete reaction system and assay procedures are described in the Materials and Methods. Data represent mean values ± S.E.M. from triplicate assays on each of two separate experiments.

DISCUSSION

As outlined in the Introduction, C_3 species metabolize a significant amount of recently fixed carbon through the glycollate-glycerate pathways; the partial reactions of which span three organelles (Scheme 1). The highly compartmented nature of these reactions imply a high degree of co-ordination between enzymic activity and flow of intermediates between these organelles. Studies of mature leaves obtained from greenhouse-grown plants, exposed to sequential light and dark regimes, have led to the proposal that this carbon flow is tightly coupled (Tolbert and Ryan, 1976). However the *de novo* synthesis of key enzymes, and the considerable degree of morphological change these organelles undergo, in response to illumination (Introduction), imply a gradual co-ordination of the partial reactions during the greening period. Thus during this developmental phase it is not unreasonable to suppose that some of these reactions may serve to satisfy the many other metabolic processes which may be occurring. The present study supports this contention, and indicates that differences may exist between glycollate metabolism in greening barley leaves and that which generally occurs in mature leaves of C_3 species. In the following discussion, the nature of both folate and glycollate metabolism in greening barley leaves will be considered, particular emphasis being placed on the possible role of glyoxylate as an intermediate in 10-formyltetrahydrofolate synthesis. In addition, carbon flow in the glycollate pathway, and via C-1 metabolism, will be assessed in relation to other metabolic processes which accompany the greening process.

Glycollate metabolism and the glycollate pathway, in barley leaves during greening.

The overall path of carbon in the glycollate pathway is well established in mature leaves of C_3 species, although the immediate substrate of photorespiratory CO_2 is still the subject of some debate (Halliwell, 1978). However, a comparison between data obtained in the present studies, and that from investigations of mature leaves, carried out under similar conditions, reveal some striking differences.

Pulse-feeding of [2-^{14}C]glycollate to mature wheat leaves (Wang and Burris, 1963) resulted in 10% and 50% of the label in the amino acid fraction, being recovered in glycine and serine respectively. A similar finding has been reported for [1-^{14}C]glycollate metabolism in mature pea (Miflin *et al.*, 1966) and barley leaves (Tamàs and Bidwell, 1971). These data imply that mitochondrial serine hydroxymethyltransferase activity is high in these tissues, and are consistent with a functional glycollate pathway in both peroxisomes and mitochondria (Scheme 1). In the present studies, the rapid metabolism of glycollate to glycine (Table 11), and the presence of glycollate oxidase activity (Figure 2) argues for an active peroxisomal component of the glycollate pathway in barley leaves, greened for 24 hrs. However, the low rates of serine labelling, either from [2-^{14}C]glycollate (90% recovered in glycine, 4% in serine; Table 11) or from both [1-^{14}C]- and [2-^{14}C]glycine (less than 2% recovered in serine; Table 12) raises questions concerning the activity of serine hydroxymethyltransferase in these leaves. These latter data are particularly interesting considering the rapid metabolism of glycine to serine, or products of serine metabolism, shown by studies of mature leaves from several C_3 species (Wang and Burris, 1963; Miflin *et al.*, 1966; Waidyanatha *et al.*, 1975). While the present studies have

not assessed the mitochondrial serine hydroxymethyltransferase directly, leaf homgenates had very low transferase activity (0.06 µmoles/hr/mg chlorophyll), when compared to either glycollate oxidase at the same greening stage (75 µmoles/hr/mg chlorophyll; Figure 2), or to recent literature values for the transferase (30-50 µmoles/hr/mg chlorophyll), obtained for homogenates of mature leaves of several C_3 species (Woo, 1979).

As outlined in the Introduction, there is good evidence that serine synthesis in the mitochondria is coupled to glycine synthetase activity. Indeed, it may be that the cleavage of glycine, which occurs in this step (Scheme 1), is fundamental to the operation of the glycollate pathway (Tolbert and Ryan, 1976). However, although barley leaves, which had been greened for 24 hrs, demonstrated this activity (Table 12), its rate again appears to be quite low.

It is apparent from these data, that while glycine is an important product of glycollate metabolism in barley leaves after 24 hrs of greening, these leaves do not have the ability to rapidly metabolize glycine through the mitochondrial reactions of the glycollate pathway. The presence of a fully functional glycollate pathway in mature leaves of this species (Tamàs and Bidwell, 1971) therefore suggests a gradual activation and co-ordination of this pathway during the greening period. In this regard, it is noteworthy that a 12 hr illumination period was required before appreciable amounts of recently fixed carbon entered the glycine-serine pool, in a study of greening barley leaves (Tamàs *et al.*, 1970). Unfortunately, as these latter workers did not separate data for glycine and serine comparisons with the present work are somewhat limited.

Formate metabolism in greening barley leaves: the nature of the processes involved

In a recent review, Halliwell (1978) has indicated that there are two major routes for formate metabolism in leaf tissues; one involving oxidation, and the other activation as a C-1 unit. Radioisotope feeding studies (Tolbert, 1955; Cossins and Sinha, 1965; Bowman and Rohringer, 1970; Kent, 1972; Foo and Cossins, 1978; Hansen and Nelsen, 1978) have shown that a wide variety of green tissues rapidly metabolize formate into typical products of folate metabolism; such as serine, methionine, glycine and adenine. These observations, together with the wide occurrence of 10-formyltetrahydrofolate synthetase in mature leaves, and photosynthetic microorganisms (Iwai *et al.*, 1967; Lor and Cossins, 1978) have led to the proposal that in such species, the major route for 10-formyltetrahydrofolate synthesis is via formate activation. A similar conclusion may be drawn for the C_3 and C_4 species examined in the present studies. Here, the amino acid labelling profiles, resulting from [^{3}H]formate metabolism (Table 6) and the presence of an active 10-formyltetrahydrofolate synthetase (Figure 4; Appendix C) provide supporting evidence. Furthermore, in barley, the inhibitory effect of aminopterin on product labelling from [^{3}H]formate (Table 8), and on the specific radioactivities of serine and glycine (Table 9) demonstrates the involvement of folate-dependent enzymes in the pathway of formate metabolism. An interesting corollary to these conclusions is provided by a recent study (Acedo, 1979) where it was shown that exogenous formate greatly stimulated the growth of an *Arabidopsis* mutant possessing low levels of several key folate-dependent enzymes.

The low $^{3}H/^{14}C$ ratios, obtained in the comparative labelling studies (Table 4), indicate that oxidation of formate also occurs in barley. Additionally, the nature of the labelled products and the higher ratios for etiolated leaves, suggest that this oxidation generates CO_2 which is re-fixed in the light, and then metabolized via the Calvin cycle. Two major routes for formate oxidation to CO_2 in leaves have been proposed. The first involves NAD^+-coupled formic dehydrogenase, which has been demonstrated in homogenates from a variety of plant tissues (Cossins and Sinha, 1965). In leaves, this enzyme is believed to be almost entirely mitochondrial (Halliwell, 1974; 1978), and of low activity (Halliwell, 1974, 1978; Grodzinski, 1979). Halliwell (1974), has proposed a second route for formate oxidation in leaves, involving the peroxidatic action of catalase. This reaction has been shown to occur in leaf peroxisomes (Halliwell, 1974; Grodzinski, 1979) and uses H_2O_2 derived from glycollate oxidation (Halliwell, 1978).

While the present studies clearly indicate that greening barley leaves effectively oxidize exogenously supplied formate, the relative contributions of the above reactions to this process have not been assessed. Additionally no information is available as to the nature and extent of oxidation of endogenous formate pools, which may be generated in barley during the greening period. However it is possible that only low rates of oxidation of these pools may be occurring since Grodzinski (1979) has shown that leaf homogenates of spinach-beet contain low NAD^+-formic dehydrogenase activity, as compared to 10-formyl-tetrahydrofolate synthetase, and has argued that the major fate of formate, generated in leaf peroxisomes, involves the latter enzyme.

Possible interrelationships between glycollate and folate metabolism in greening barley.

There is considerable debate in the current literature (Introduction) concerning the role of glyoxylate in glycollate metabolism, as this intermediate may be situated at a key branch-point in the glycollate pathway (Scheme 3). Indeed, recent studies by Grodzinski (1978) and Oliver (1979) indicate that a significant proportion of the photorespiratory CO_2 released from glycollate, arises via glyoxylate decarboxylation. Since formate is also generated by this reaction (Halliwell and Butt, 1974; Scheme 3), these conclusions are of particular interest to the present studies in the light of the increasing rate of 10-formyltetrahydrofolate synthesis which occurs as the etiolated barley leaf greens (Figures 4 and 9).

While an analogous carbon flow from glycollate → glyoxylate → formate → 10-formyltetrahydrofolate can be implied from the greening data (Figures 2,3,4 and 9), the inhibitory effect of αHPMS on formate metabolism *in vivo* (Tables 16 and 17), and on the activity of 10-formyltetrahydrofolate synthetase in leaf homogenates (Table 18) provide good evidence for a relationship between glycollate metabolism and 10-formyltetrahydrofolate synthesis in barley. A similar conclusion has been drawn from a study of folate metabolism in *Euglena gracilis* (Lor and Cossins, 1978) where αHPMS treatment greatly reduced 10-formyltetrahydrofolate synthetase activity, and the pool sizes of metabolically important folates.

The magnitude of this carbon flow from glycollate to 10-formyltetrahydrofolate is difficult to assess. The lack of appreciable dilution in the formate competition experiments (Table 15) clearly

questions the involvement of formate in the path of carbon from glycollate and suggests that the major flow from glycollate is via glycine transaminase (Scheme 3). This does not negate glyoxylate decarboxylation however, since a similar finding has been reported for spinach-beet peroxisomes (Grodzinski, 1979), where significant formate production by glyoxylate decarboxylation has been reported (Halliwell and Butt, 1974; Grodzinski, 1979). Indeed, viewed quantitatively the present enzyme data (Figures 2 and 4) suggest that glyoxylate decarboxylation and 10-formyltetrahydrofolate synthesis can account for about 20% of glycollate metabolized in barley during the first two days of greening.

An interesting parallel to these present studies is provided by the data of Lor and Cossins (1978), who have suggested that in *Euglena gracilis*, a product of glycollate metabolism, conceivably formate, may be necessary to maintain high 10-formyltetrahydrofolate synthetase levels. The inhibitory effects of αHPMS (Tables 16 and 17), and the effect of exogenous formate on the pools of formate related amino acids (Table 15), indicate that a similar situation may also prevail in barley. While these possibilities have not been examined further, it is interesting to note that formate is known to induce 10-formyltetrahydrofolate synthetase in *Micrococcus aerogenes* (Whiteley, 1967). The possible regulatory role of formate, suggested in these studies, is clearly an area worthy of further study.

Production of labelled serine and glycine during [^{3}H]formate feeding

In keeping with previous studies (Tolbert, 1955; Cossins and Sinha, 1965; Bowman and Rohringer, 1970; Kent, 1972; Calmés and Viala,

1978; Foo and Cossins, 1978), serine was a major labelled product of formate metabolism in all species examined in this study (Table 6). While serine hydroxymethyltransferase activity can be implied from these data, the inhibition of serine labelling by INH (Table 10) provides supporting evidence (Miflin *et al.*, 1966; Oliver, 1979) for the involvement of this enzyme in barley leaves. This finding is somewhat surprising considering the apparent low activity of this enzyme in leaf homogenates (Table 13), and the results from previously discussed feeding experiments (Table 11). It should be noted however that serine hydroxytransferase activity was assayed by measurement of 5,10-methylenetetrahydrofolate production (Materials and Methods). Thus if the enzyme is not freely reversible *in vivo*, labelling could arise during formate feeding. In addition, serine may also arise at a site which is independent of the glycollate pathway. This possibility is supported by reports that serine hydroxymethyltransferase activity is commonly cytosolic (Kisaki *et al.*, 1971a; Woo, 1979) and chloroplastic (Shah and Cossins, 1970; Kisaki *et al.*, 1971a; Woo, 1979) in leaves of C_3 species. Involvement of a chloroplastic enzyme is conceivable from the present data (Table 12) as exogenous glycine, which was metabolized at low rates, does not readily penetrate the inner envelope membrane of C_3 plant chloroplasts (Halliwell, 1978). Formate on the other hand is readily taken up by chloroplasts (Halliwell, 1978), and might therefore play a key role in serine synthesis, particularly during the greening period when considerable membrane synthesis and morphological development is occurring (Bradbeer *et al.*, 1977). Clearly, the foregoing arguments are somewhat speculative in nature and more detailed studies on the

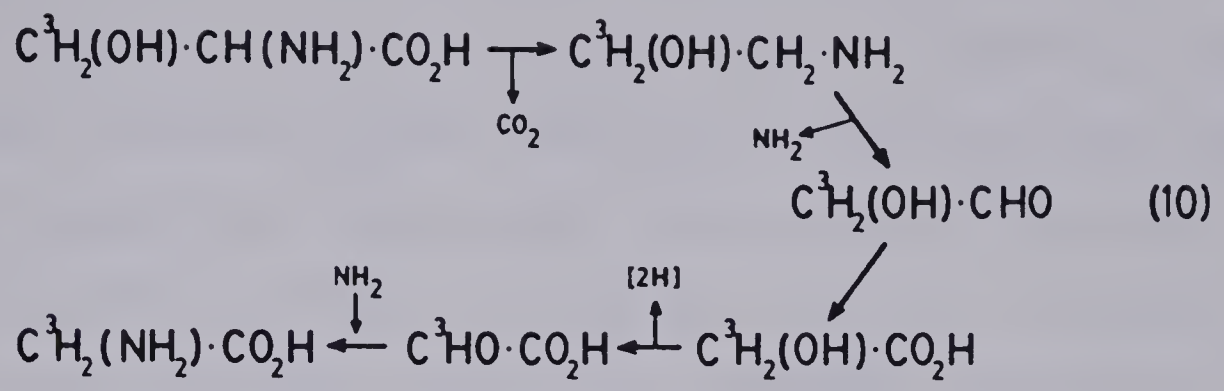
nature of serine hydroxymethyltransferase and it compartmentation in greening barley leaves, are needed.

As outlined in the Results, glycine is an important product of formate metabolism in barley, particularly during the initial 24 hr greening period when its rate of labelling is maximal (Figure 9). In addition to barley, this amino acid was also labelled by ^{3}H in the other C_3 species examined (Table 6). The data for broad bean are contrary to that reported by Kent (1972) who used similarly grown greenhouse material. However in his work Kent (1972) infiltrated the leaves with [^{3}H]formate in the dark for 1 hr, prior to illumination and subsequent analysis. Considering the long dark period, during which significant formate metabolism must occur (Table 7; Cossins and Sinha, 1965; Bowman and Rohringer, 1970; Foo and Cossins, 1978) it is difficult to assess the relevance of this work to that in the present study.

Glycine labelling from [^{14}C]formate has been reported in a number of studies of mature leaves (Cossins and Sinha, 1965; Bowman and Rohringer, 1970; Calmés and Viala, 1978), particularly when feeding was carried out in the light. In these studies, some ^{14}C incorporation may have occurred as a result of $^{14}CO_2$ fixation (Table 4; Cossins and Sinha, 1965; Halliwell, 1978). However, as noted in the Results, the use of [^{3}H]formate in the present studies precluded this possibility and permitted some assessment of the generation and subsequent metabolism of 10-formyltetrahydrofolate.

There are several plausible routes for the incorporation of this C-1 unit into glycine. For example Sinha and Cossins (1964) suggested that serine may give rise to glycine through the action of a glycine-

ethanolamine cycle, equation 10. While it is theoretically possible to



generate tritiated glycine by this route, the overall low rate of [3-^{14}C]serine metabolism (Table 14), as indicated by the large amounts of labelled serine remaining after the feeding period, and the small degree of glycine labelling from this precursor (Table 14) argues against this being an important route for glycine generation *in vivo* in greening barley leaves. Both wheat (Bowman and Rohringer, 1970) and barley leaves (Hanson and Nelsen, 1978) incorporate [^{14}C]formate into betaine via serine, ethanolamine and choline. As betaine can be oxidized to glycine (Greenberg, 1969) this may represent a potential route for glycine synthesis. However, in this pathway the C_3 of serine becomes the C_1 of glycine, and this is accompanied by the elimination of ^{3}H due to ionization.

It is also possible to generate glycine from 5,10-methylene-tetrahydrofolate by a reversal of glycine cleavage (equation 4, P. 10). This reaction, catalyzed by glycine synthase, has been shown to be reversible in the mitochondria of pea cotyledons (Clandinin and Cossins, 1972) and in mammalian liver (Kikuchi, 1973), but in mature leaves of C_3 species which form serine from glycine, its direction appears to favour cleavage (Halliwell, 1978). However, the inhibitory effect of INH on glycine labelling from [^{3}H]formate (Table 10), suggests that the reverse reaction may play an important role in glycine generation in greening

barley leaves. In this reaction ^{3}H, derived from [^{3}H]formate, can enter the $C^{3}H_{2}NH_{2}$ group of glycine (Kikuchi, 1973). It should be noted however, that the observed glycine labelling may not be the direct result of *de novo* synthesis, but rather reflect a contribution of the reverse reaction to overall equilibrium. This appears unlikely as the rates of cleavage in this tissue were low (Table 12). Furthermore the kinetics of glycine labelling (Figure 6) and the high percentage of incorporation of ^{3}H in glycine relative to other labelled amino acids throughout the greening period (Table 6; Figure 9) suggest that a net *de novo* synthesis was occurring. If this is the case, then glycine would conceivably exist in pools which are actively turning over, thereby creating a demand for continued synthesis during greening. Such a turnover in greening barley can be implied from the considerable increases in glycine pool size observed in response to the INH (Table 10) and aminopterin (Table 9) treatments. There is also good evidence for a rapid turnover of free glycine in barley leaves during greening from the work of Hendry and Stobart (1978). In fact, these workers have suggested that in the early stages of greening, when conditions do not favour high rates of photorespiration, glycine may play an important role in both δ-aminolaevulinic acid and chlorophyll biosynthesis (Hendry and Stobart, 1977b; Stobart and Hendry, 1978). Interestingly, this observation may shed some light on the parallel rates of incorporation of ^{3}H into glycine (Figure 9) and the ether soluble fraction (Figure 8), observed in the present studies.

From the present studies it is apparent that glycine becomes increasingly important as etiolated barley leaves green. During this

period, this amino acid can be generated from both the partial reactions of the glycollate pathway and from formate via C-1 metabolism. The relative contributions of these processes to net glycine synthesis, and the dynamics of their compartmentation, represent important areas for future research.

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APPENDIX A

AN ISCO SPECTRORADIOMETER ANALYSIS OF LIGHT QUALITY IN THE CONTROLLED ENVIRONMENT CHAMBER USED IN THIS STUDY.

Wavelength (nm)	Irradiance intensity $\mu W\ cm^{-2}\ nm^{-1}$	Wavelength (nm)	Irradiance intensity $\mu W\ cm^{-2}\ nm^{-1}$
380	0.1	800	5.1
400	0.2	850	4.2
425	0.7	900	2.3
450	1.4	950	2.3
475	2.0	1000	2.3
500	2.9	1050	2.5
525	3.8	1100	2.7
550	8.6	1150	2.2
575	18.0	1200	1.5
600	17.6	1250	3.0
625	12.0	1300	4.1
650	7.6	1350	2.5
675	4.9	1400	1.0
700	3.9	1450	0.6
725	4.0	1500	1.3
750	4.8	1550	2.0

Growthchamber lamps were a combination of cool white fluorescent and 100 watt incandescent. Irradiance intensity was measured at 40 cm from the lamps.

APPENDIX B

CHANGES IN SOLUBLE AMINO ACID POOLS IN ETIOLATED AND GREENING BARLEY LEAVES.

All results are expressed as μmoles/g fresh wt. leaf tissue

T - trace amount, <0.05 μmoles/g fresh wt.

Pro, Cys, Phe were detected as trace amounts in both greened and etiolated leaves. Arg was present as a trace amount in greened leaves. Met was not detected.

The procedures followed were those outlined in the Materials and Methods. Six-day etiolated leaves were either greened or held in the dark as controls. Samples were taken throughout a four day treatment period and individual amino acid pools determined. Data represent mean values ± S.E.M., obtained from duplicate determinations on each of three separate experiments.

soluble amino acids	Days greened				
	6.5	7	8	9	10
Lys	0.41 ± *0.04*	0.23 ± *0.03*	0.21 ± *0.02*	0.28 ± *0.03*	0.21 ± *0.02*
His	0.52 ± *0.07*	0.23 ± *0.02*	0.14 ± *0.01*	T	T
Asn	5.73 ± *0.42*	2.00 ± *0.05*	1.13 ± *0.21*	0.15 ± *0.03*	0.18 ± *0.03*
Gln	1.16 ± *0.19*	0.81 ± *0.13*	1.93 ± *0.14*	1.77 ± *0.22*	1.47 ± *0.29*
Asp	2.81 ± *0.71*	0.91 ± *0.08*	0.98 ± *0.11*	1.06 ± *0.12*	0.88 ± *0.16*
Thr	0.89 ± *0.08*	0.38 ± *0.08*	0.63 ± *0.01*	0.69 ± *0.13*	0.63 ± *0.13*
Ser	1.23 ± *0.24*	0.77 ± *0.06*	1.41 ± *0.22*	1.39 ± *0.37*	1.12 ± *0.19*
Glu	2.25 ± *0.24*	1.27 ± *0.11*	2.89 ± *0.27*	3.22 ± *0.40*	3.27 ± *0.63*
Gly	0.67 ± *0.05*	0.42 ± *0.05*	0.54 ± *0.08*	0.45 ± *0.08*	0.47 ± *0.14*
Ala	1.97 ± *0.38*	0.90 ± *0.01*	2.30 ± *0.11*	1.83 ± *0.18*	2.25 ± *0.41*
Val	1.03 ± *0.09*	0.39 ± *0.02*	0.34 ± *0.02*	0.49 ± *0.08*	0.36 ± *0.06*
Ile	0.31 ± *0.01*	0.12 ± *0.02*	0.09 ± *0.01*	0.14 ± *0.03*	0.12 ± *0.04*
Leu	0.14 ± *0.02*	0.08 ± *0.02*	0.08 ± *0.01*	0.16 ± *0.02*	0.13 ± *0.04*

APPENDIX B - CONTINUED

soluble amino acids	Days etiolated				
	6	7	8	9	10
Lys	0.54 ± *0.08*	1.02 ± *0.08*	1.17 ± *0.08*	2.21 ± *0.10*	1.22 ± *0.08*
His	0.77 ± *0.17*	0.96 ± *0.07*	1.27 ± *0.10*	1.59 ± *0.09*	0.95 ± *0.07*
Arg	T	0.28 ± *0.02*	0.35 ± *0.04*	1.01 ± *0.07*	0.34 ± *0.04*
Asn	5.14 ± *0.33*	8.36 ± *0.98*	8.18 ± *1.22*	6.42 ± *0.92*	13.88 ± *2.49*
Gln	2.17 ± *0.23*	2.59 ± *0.12*	0.58 ± *0.09*	0.67 ± *0.03*	0.60 ± *0.07*
Asp	2.00 ± *0.11*	2.07 ± *0.24*	2.18 ± *0.33*	3.86 ± *0.55*	5.47 ± *0.98*
Thr	1.06 ± *0.08*	1.10 ± *0.17*	1.15 ± *0.23*	1.41 ± *0.07*	2.16 ± *0.21*
Ser	2.24 ± *0.42*	2.17 ± *0.11*	2.06 ± *0.21*	2.47 ± *0.08*	3.19 ± *0.42*
Glu	2.27 ± *0.15*	2.22 ± *0.11*	2.00 ± *0.24*	2.23 ± *0.09*	2.54 ± *0.29*
Gly	0.75 ± *0.03*	0.82 ± *0.02*	0.60 ± *0.06*	0.74 ± *0.05*	0.96 ± *0.17*
Ala	6.47 ± *0.96*	4.99 ± *0.08*	5.11 ± *0.43*	6.15 ± *0.21*	7.24 ± *1.40*
Val	1.91 ± *0.19*	1.63 ± *0.44*	2.14 ± *0.26*	2.50 ± *0.02*	3.82 ± *0.61*
Ile	0.59 ± *0.07*	0.82 ± *0.11*	0.73 ± *0.08*	0.90 ± *0.03*	1.51 ± *0.28*
Leu	0.42 ± *0.04*	0.62 ± *0.11*	0.51 ± *0.05*	0.62 ± *0.01*	0.84 ± *0.10*
Tyr	T	T	T	T	0.23 ± *0.05*

APPENDIX C

ACTIVITY OF 10-FORMYLTETRAHYDROFOLATE SYNTHETASE AND GLYCOLLATE OXIDASE IN GREENED AND ETIOLATED LEAVES OF DWARF BEAN AND CORN.

	dwarf bean		corn	
	greened	etiolated	greened	etiolated
Soluble protein				
mg/g fresh wt.	31.7 ± *1.3*	37.4 ± *2.1*	13.2 ± *0.1*	11.7 ± *0.7*
Enzyme activity				
units/g fresh wt.				
Synthetase	213.6 ± *8.5*	195.5 ± *15.8*	127.2 ± *11.9*	78.7 ± *2.4*
Oxidase	1921.5 ± *26.6*	1005.7 ± *23.1*	295.9 ± *3.1*	*n.d.*

n.d. - not detected

1 unit = 1 nmole product/min

Growth and treatment periods were as outlined in the Materials and Methods, and Table 6. The complete reaction systems and assay procedures are described in the Materials and Methods. Data represent mean values ± S.E.M.,obtained from triplicate determinations on each of two separate experiments.